ACETYLCHOLINESTERASE AND ACETYLCHOLINE RECEPTOR

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Our previous studies on cationic and uncharged substrates and reversible aliphatic and aromatic inhibitors led to the conclusions (i) that the subsite at which the trimethylammonio group of acetylcholine (AcCh) binds to acetylcholinesterase (AcChE) is not "anionic" but uncharged "trimethyl" in character; (ii) contiguous to it are aromatic aminoacid sidechains; (iii) the cationic and uncharged groups bind at the same site; and (iv) uncharged reagents would more specifically characterize the active site. Bromopinacolone, $(CH_3)_3CCOCH_2Br$ (BrPin), $K_{i(com)}$ = 0.2 mM, inactivated AcChE from Electrophorus with the same efficiency toward cationic and uncharged substrates.

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AcChE was isolated from T. nobiliana, k_{cat} = 5.6 x 10^3 s^{-1}, K_m = 0.051 mM in hydrolysis of AcCh; inactivation of it by [2-^{14}C]BrPin introduced 1.9 ^{14}C per enzyme equivalent inactivated, and was retarded by 5-trimethylammonio-2-pentanone (TAP) an isostere of AcCh, with exclusion of 1 ^{14}C per enzyme unit protected. Prior inactivation by BrPin prevents reaction with [^{3}H]DFP, while inactivation by DFP does not exclude [^{14}C]BrPin. The ^{14}C label is concentrated as the pinacolonyl substituent on S of Cys-231 of the peptide beginning at Ala-222. This was confirmed by (i) study of S-pinacolonylcysteine, (ii) by blocking the sequencing by orthophthalaldelyde, and (iii) inactivation of the enzyme by 2,2′-dipyridyldisulfide (2-PDS, $K_{i(com)}$ = 0.03 mM). Inactivation by 2-PDS prevents reaction with [^{14}C]BrPin. 2-PDS does not inactivate AcChE from Electrophorus and is a weak mixed inhibitor of it.

Inactivation of the AcChEs by phenacyl bromide (PhABr) is efficent, is retarded by phenyltrimethylammonium ion (PTA), and prevents reaction with [^{14}C]BrPin. Inactivation by methyl benzenesulfonate is retarded by 3-acetylpyridine and 2-dimethylaminopyridine but not by PTA or TAP.

Study of reversible inhibition by tri- and tetraalkylammonio compounds of hydrolysis by AcChE from Electrophorus of substrates of varied reactivity indicates that noncompetitive inhibition and inhibition by substrates generally arise from binding to enzyme-substrate complex rather than to acylenzyme.

Reversible inhibition by substituted pyridines has been studied. Dimethylaminopyridines, $K_{i(com)}$ ~ 0.1 mM, without permanent positive charge, bind as strongly as N-methylpyridiniuom ion.

FOREWORD

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SUMMARY

A. Our previous studies on hydrolysis by AcChE from Electrophorus of varied cationic and uncharged substrates and on inhibition by varied cationic and uncharged reversible inhibitors led us to the conclusion that the site at which the trimethylammonio group of acetylcholine (AcCh) binds is not "anionic," as was generally accepted, but hydrocarbon in character, better termed "trimethyl," and would be more specifically explored by uncharged reagents, such as 1-bromopinacolone (BrPin). Study of inhibition by derivatives of benzene led us to the view that aromatic amino acid side chains contiguous to the trimethyl subsite formed part of the active site and would specifically bind reagents such as phenacyl bromide (PhABr), methyl benzenesulfonate (MBS) and bromoacetyl pyridine (BAPy). These conclusions as to the character of the active site have been proven valid by the studies reported herein and by recent X-ray diffraction studies of AcChE from T. californica (26). Our initial kinetic studies were carried out with commercially available AcChE from Electrophorus; labeling with BrPin was carried out on AcChE from T. nobiliana, isolated in our laboratory.

B. 1-Bromo-2-[^{14}C]-pinacolone, $(CH_3)_3C^{14}COCH_2Br$ ([^{14}C]BrPin), was prepared from 1-[^{14}C]-acetyl chloride and tert-butylmagnesium chloride with cuprous chloride catalyst, followed by bromination. It was examined as an active-site-directed label for AcChE. AcChE was isolated from T. nobiliana; it has k_{cat} = (4.00 ± 0.04) x 10^3 s^{-1}, K_m = 0.055 ± 0.008 mM in hydrolysis of acetylthiocholine (AcSCh), and k_{cat} = (5.6 ± 0.2) x 10^3 s^{-1}, K_m = 0.051 ± 0.003 mM in hydrolysis of AcCh. BrPin, binding in the trimethyl cavity, acts initially as a reversible competitive inhibitor, K_i = 0.18 mM, and then inactivates the enzyme, k_2 = 1.8 x 10^{-4} s^{-1}. Introduction of ^{14}C from [^{14}C]BrPin into this AcChE at pH 7.0 was followed by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), autoradiography and scintillation counting, in the absence and presence of 5-trimethylammonio-2-pentanone (TAP), a competitive inhibitor (K_i = 0.075 ± 0.001 mM), isosteric with AcCh; 1.8-1.9 ^{14}C was incorporated per inactivated enzyme unit at 50% inactivation. TAP retarded inactivation by [^{14}C]BrPin, and prevented introduction of 0.9-1.1 ^{14}C per unit of enzyme protected. Prior inactivation of AcChE by BrPin prevents reaction with [^{3}H]-diisopropyl fluorophosphate ([^{3}H]DFP). Prior inactivation by DFP or [^{3}H]DFP does not prevent reaction with [^{14}C]BrPin, and this subsequent reaction with BrPin does not displace the [^{3}H]-moiety. Decamethonium (DeMe) and 3-trimethylammonioacetophenone (TMAAPh) protect as efficiently as TAP. Phenyltrimethylammonium ion (PTA) and 3-dimethylammonio-N-methylpyridinium ion (DMMPy) appear to offer less protection against BrPin.

Acetylcholinesterase from T. nobiliana, was treated with [^{14}C]BrPin or with BrPin, and aliquots, so treated and without such inactivation, were denatured by guanidinium, reduced by dithiothreitol (DTT), alkylated by iodoacetate and digested with trypsin, and the tryptic digests were fractionated by reversed-phase HPLC. Inactivation by BrPin or [^{14}C]BrPin caused displacement of one peptide, that beginning at Ala-222. TAP retarded the inactivation and decreased the quantity of displaced peptide. Sequencing of the displaced peptide by Edman degradation was carried out by Professor J. B. Cohen, Washington University School of Medicine. The ^{14}C label was found to be associated with Cys-231 in an amount proportional to the amount of this peptide. Identification of Cys-231 was confirmed by comparison with synthesized S-pinacolonylcysteine and study of effects of blocking the sequencing by treatment with ortho-phthalaldehyde (OPA). [^{14}C]BrPin reacts with the -SH of Cys-231 and this reaction prevents hydrolysis of substrates in the active site of AcChE of T. nobiliana.

Presence of Cys-231 at the active site was confirmed by analysis of the effects of thiol-specific reagents; 2,2′-dipyridyldisulfide (2-PDS; acts initially as a competitive inhibitor, K_i = 0.039 mM, and then inactivates the enzyme, k_2 = 5.0 x 10^{-4} s^{-1}, and this inactivation also is retarded by TAP. 5,5′-Dithiobis(2-nitrobenzoic)acid (DTNB) and iodoacetamide (IAm) were less effective than 2-PDS, and iodoacetate did not inactivate AcChE from T. nobiliana. Phenylmercuric chloride (PMc) did inactivate it. Prior inactivation by 2-PDS prevents subsequent reaction of [^{14}C]BrPin in the active site. 2-PDS is a weaker, mixed inhibitor of AcChE from Electrophorus, $K_{i(com)}$ 3.2 mM, $K_{i(nonc)}$ 1.2 mM, and does not inactivate it or that from human erythrocyte, indicating that they do not appear to possess active site -SH. BrPin does inactivate these enzymes, alkylating another residue. BrPin and 2-PDS do not inactivate butyrylcholinesterase (BuChE) from human or horse serum.

In experiments in collaboration with Dr. Clarence Broomfield, Aberdeen Proving Ground, prior inactivation by BrPin prevented incorporation of ^{3}H on subsequent treatment with [^{3}H]-soman, just as with [^{3}H]DFP. Prior inactivation by soman decreased introduction of [^{14}C] from [^{14}C]BrPin, while prior inactivation by [^{3}H]DFP had not.

AcChE has been isolated from electric organ of Electrophorus. It is not inactivated by 2-PDS.

AcChEs from T. nobiliana and Electrophorus were inactivated by [^{3}H]DFP, and labeled Ser-200 containing peptides were isolated and sequenced, and were found essentially identical with that proposed for the Ser-200 region of AcChE from T. californica.

C. PhABr, ($K_{i(com)}$ 1.6 mM, $K_{i(nonc)}$ 1.4 mM) at 1.6 mM inactivated AcChE from Electrophorus with $t_{1/2}$ ~ 6 hr; at 0.25 mM it inactivated AcChE from T. nobiliana ($K_{i(com)}$ 1.5 mM, $K_{i(nonc)}$ 0.9 mM) with $t_{1/2}$ ~ 10 min. PTA protected both enzymes against inactivation by PhABr. In study with enzyme from Electrophorus, N-methylpyridinium ion (NMPy), 2-dimethylaminopyridine (2-DMAPy), 5-N,N-diethyl-N-n-butylammonio-2-pentanone (DEBAP) and TAP offered less protection while tetramethylammonium ion (TeMA) offered none. Compounds, each occupying only the trimethyl and aryl binding sites, may be bound simultaneously. Prior inactivation by PhABr excluded [^{14}C]BrPin from the active site of T. nobiliana. Synthesis of PhABr on a 100-μmol scale has been worked out in anticipation of preparation of radioactive material. Treatment of BrPin and PhABr inactivated Torpedo enzyme with tritiated borohydride ([^{3}H]BH_4^-) proved less promising as a method of specific active-site labeling.

D. Methyl benzenesulfonate, ($K_{i(com)}$ 5.9 mM, $K_{i(nonc)}$ 21 mM,) at 1.5 mM inactivated AcChE from Electrophorus with $t_{1/2}$ ~ 1 hr. This inactivation is strongly retarded by N-methylacridinium ion (MACr), 3-acetylpyridine (3-AcPy) and 2-DMAPy, less well by NMPy and propidium; TAP and PTA do not protect. Its synthesis on a 100-μmol scale has been worked out. Prior inactivation by MBS prevented [^{14}C]BrPin from reacting in the active site of Torpedo AcChE.

E. Noncompetitive inhibition and inhibition by substrate: To assess the relative importance of binding to enzyme-substrate complex (E·S) and to acetylenzyme (EA), noncompetitive inhibition has been studied in hydrolysis by AcChE of cationic and uncharged substrates - AcCh, 3,3-dimethylbutyl acetate (DMBAc), n-butyl acetate (n-BAc), 2-(methylammonio)ethyl acetate (MAAc), 2-(N,N-diethyl-N-n-butylammonio)ethyl acetate (DEBAAc) and 2-(methylsulfonyl)ethyl acetate (MSAc). For the N-trimethylquaternary ions related to AcCh, i.e. TEMA, choline and choline ethyl ether, noncompetitive inhibition ($K_{i(nonc)}$) is more favorable with the slower substrates than with AcCh, i.e., when E·S > EA, and is attributed to formation of enzyme-substrate-inhibitor complexes, E·S·I′. Noncompetitive inhibition by tetraethyl-, tert-butyl- and isopropylammonium ions, and acetamidocholine and its lower dimethyl analogue is also attributed to E·S·I′ complexes. Peripheral binding of these inhibitors decreases acylation more than deacylation. Some tertiary dimethylammonio ions have more favorable $K_{i(nonc)}$ values with AcCh, decreasing deacylation more than acylation. The substrate 2-N,N-diethyl-N-n-butylammonioethyl acetate DEBAAc is a more effective noncompetitive than competitive inhibitor in hydrolysis of AcCh, indicating that it binds more strongly in a peripheral site than in the active site of the free enzyme. In its hydrolysis by AcChE, it acts as its own noncompetitive inhibitor by this non-productive binding. Formation of E·S·I′ complexes is a general characteristic of hydrolysis by AcChE, and

decrease in rates at high concentrations of AcCh and related substrates is attributed to peripheral regulatory site binding, formation of E·S·S′ complexes, rather than to binding to the EA.

F. Derivatives of pyridine, like those of benzene, generally show mixed reversible inhibition. Pyridine binds more strongly than benzene, and binding is increased by hydrocarbon substituents and by electron withdrawing substituents, but with smaller effect than in benzenes. Electron-donating substituents increase binding but decrease that of benzene. Dimethylamino substituents greatly increase binding, to $K_{i(com)}$ 0.05-0.17 mM, similar to that of NMPy, without the presence of a permanent cationic charge, and thus combine strong binding with possible high membrane permeability.

Values of noncompetitive inhibition of benzene and pyridine derivatives, unlike those of inhibitors structurally related to AcCh, do not correlate simply with substrate reactivity. Binding of substituted pyridines with AcChEs from Electrophorus and T. nobiliana were compared; no uniform relation was found.

3-Bromoacetylpyridine inactivates AcChE from Electrophorus similarly to PhABr. Partial protection is offered by PTA, 3-AcPy, and NMPy; little protection is given by TMAAc, TAP, TMA, DMAPy and DeMe; tubocurarine does not protect.

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I. Statement of the Problem Under Study

A. The Trimethyl Site

It had long been accepted that the active site of AcChE has an anionic subsite to which the cationic groups of AcCh are attracted. From kinetic studies of cationic and uncharged substrates and reversible inhibitors, we proposed that the subsite is uncharged, complementary to the hydrocarbon surface of the trimethylammonio groups, better termed "trimethyl," than "anionic" and more specifically studied by uncharged reagents. We chose 1-bromopinacolone, $(CH_3)_3CCOCH_2Br$ (BrPin), as the active-site-specific alkylating agent, and undertook to prepare it radioactively labeled, inactivate the enzyme with it and identify the modified active-site amino acid.

B. Aromatic Inhibitors

Study of reversible inhibition by substituted benzenes and phenols led us to the conclusion that the active site includes an aryl binding subsite, contiguous with the trimethyl and esteratic subsites, and probably comprising aromatic side chains. Thus we are studying aryl alkylating agents, phenacyl bromide, (PhABr) methyl benzenesulfonate (MBS) and bromoacetylpyridine (BAPy), as active-site-directed inactivating and labeling agents. Views of improved aryl reactivating agents and medicinals are developed.

C. Noncompetitive Inhibition

Noncompetitive inhibition by substituted ammonium ions, and by extension, inhibition by high concentration of acetylcholine (AcCh) have been attributed to binding to acetylenzyme (EA), forming EA·I and EA·S complexes. Comparison of inhibition by a series of homologous tri- and tetrasubstituted reversible inhibitors structurally related to AcCh led us to the conclusion that noncompetitive inhibition by such tetrasubstituted compounds, and in particular by AcCh itself, arises from binding to the enzyme-substrate complex, E·S, forming E·S·I′ and E·S·S′ complexes.

II. Background and Review of Appropriate Literature

A somewhat more detailed description has been given in the Midterm Report (1).

A. The Trimethyl Site

1. Substrates

The "anionic" site was early proposed to attract, orient, and lead to high reactivity of AcCh and related cationic substrates, and to strong binding of related inhibitors (2-4).

It was recognized that the N-methyl groups were essential for strong binding (4,5), and suggested that there is no negative charge in the purported anionic site (6). The existence and importance of the "anionic" site remained widely accepted and supported (7-9), despite the high rate constant for hydrolysis of the uncharged carbon analogue of AcCh, 3,3-dimethylbutyl acetate, $(CH_3)_3CCH_2CH_2OCOCH_3$ (DMBAc), $> 10^6\ M^{-1}\ s^{-1}$ (10).

We proposed that the positive charge in AcCh increases its intrinsic hydrolytic reactivity as to hydroxide, and this suffices to account for its greater enzymic reactivity, rate constant $5 \times 10^7\ M^{-1}\ s^{-1}$. In a study of hydrolysis of 14 β-substituted ethyl acetates, $X\text{-}CH_2CH_2OCOCH_3$, with X the four ammonio groups, H_3N^+- to $(CH_3)_3N^+$-, the analogous carbon-centered substituents, CH_3- to $(CH_3)_3C$-, and uncharged polar substituents, application of normalization factors, calculated from the effects of the substituents on alkaline hydrolysis rates, to the rates of hydrolysis by AcChE from Electrophorus, led to a linear relation between these normalized rates and the apparent molal volumes (V_{25}) of the substituents, X, eq. 1 (11), where k_2 is the acylation rate constant.

$$\log(k_{2(n)}/K_s) = a\ V_{25} + C \qquad (1)$$

In a similar study of another set of substrates, the normalized reactivity was also found to correlate with volume of uncharged and cationic β-substituents as measured by molar refraction (MR), (12). β-Substituents with polar surfaces, sulfone, $CH_3S(O_2)$-, amine oxide, $(CH_3)_2N^+(O^-)$-, and sulfoxide, $CH_3S(O)$-, led to lower reactivity than consistent with their MR values, supporting the importance of hydrocarbon surfaces.

The enzymic reactivity of these acetate esters of widely varied structure was accounted for by (i) their intrinsic hydroxide-catalyzed reactivity and (ii) the volume of the β-substituent, and thus its fit into the active site and its effect on placing the ester group at the esteratic site. Further, K_s values of AcCh and DMBAc and other cationic and uncharged pairs are similar, indicating no substantial coulombic effect (12). No specific effect of anionic charge on the enzymic hydrolysis rate, and thus no evidence for an anionic site, exists. We concluded that the subsite where the β-substituent of acetates binds is to be considered "trimethyl," complementary to this character of AcCh rather than to its positive charge. However, hydrophobicity, π, is not the property to which reactivity is related since it does not account for the high reactivity of AcCh.

2. Reversible Inhibitors

Evidence in support of the "anionic" site had been found in the decrease in binding of cationic reversible inhibitors with

decrease in pH (3), and cationic inhibitors related in structure to AcCh do bind somewhat more strongly than their uncharged analogues (13). However, this latter effect is small, corresponding to about 1 kcal/mole, much less than would be caused by interaction with a contact anionic O^- (13). As to the effect of pH, the isoelectric point of the enzyme is about 5 (14), and multiple anionic surface charges at more alkaline pH (15) may account for this effect, and also the more favorable binding of cationic species.

It is important to note further that each of the uncharged and cationic inhibitors structurally related to AcCh which we studied showed essentially the same binding constants when retarding hydrolyses of AcCh and its uncharged analogue, DMBAc. This indicates that the β-trimethylammonio and β-tert-butyl groups of the two substrates and of the related inhibitors bind at the same subsite (13) despite continuing proposals to the contrary (8).

More detailed analysis of binding at the trimethyl and esteratic subsites, and of synergism and absence of it, has been described in a publication (16) and in the Midterm Report (1). Binding of $(CH_3)_3C-$, $(CH_3)_3Si-$, $CH_3S(O_2)-$, $(CH_3)_3N^+-$, and $(CH_3)_2S^+-$ as β-substituents, of CH_3-, $-CH_2CH_2OH$ and $-CH_2CH_2OCOCH_3$ as fourth substituents, and of dimethylsulfonyl, dimethylsulfoxy and trimethylaminoxy as β-substituents were compared; Cl- and CH_3- were compared in CCl_3CHO and $(CH_3)_3CCHO$.

3. Irreversible Inhibitors

Compounds related to the substrates and reversible inhibitors, above, based on the view of the binding site as uncharged, were devised and studied as irreversible inhibitors and potential labeling agents. Inactivation by methyl methanesulfonate, and methylsulfonyl chloride and fluoride, and hydrolysis of and inactivation by chloromethylpivalate, chloromethyl acetate, trimethylammoniomethyl acetate and tert-butyl peracetate were described in the Midterm Report (1).

Primary emphasis was placed on study of inactivation and labeling by 1-bromopinacolone, $(CH_3)_3CCOCH_2Br$ (BrPin). It inactivates AcChE with the same efficiency toward hydrolysis of widely varied substrates, cationic and neutral aliphatic acetates and aryl acetates, and its inactivating action is retarded by reversible cationic inhibitors to extents appropriate to their binding constants (17). We felt that previous focus on the anionic site led to study of cationic inactivators, and that these might be attracted to anionic groups on the enzyme surface not closely related to the active site.

B. Noncompetitive Inhibition

Noncompetitive inhibition by ammonium ions related to AcCh and inhibition by high concentrations of AcCh itself were considered to arise from binding to the acetylenzyme, EA, forming EA·I and EA·S. Binding of such inhibitors to the enzyme-substrate complex E·S, forming E·S·I′ and E·S·S′, has been thought not to occur. This view was supported largely by high noncompetitive components in inhibition by tertiary ammonium ions, i.e., $(CH_3)_3NH^+$, etc., in the rapid hydrolysis of AcCh, in which much EA is present, and less noncompetitive inhibition in hydrolysis of less reactive substrates, in which less EA and much E·S is present (18,19). Since the binding subsite is not anionic and thus not particularly favored for further binding in EA, we found it appropriate to study noncompetitive components of tetrasubstituted inhibitors more closely related to AcCh, i.e., $(CH_3)_4N^+$, etc., in hydrolysis of substrates of varied reactivity. The results have been published (20) and will be summarized in a later section of this report.

C. Aromatic Inhibitors

1. Benzenes and Phenols

Cationic aromatic reversible inhibitors, phenyltrimethylammonium ion (PTA) and trimethylammoniophenol (TMAP) (21) and inactivators physostigmine (22) and neostigmine (23) have long been known, and we undertook to study substituent effects on binding of uncharged derivatives of benzene and phenol. In this we have applied the Hammett equation, $\log k/k_o = \rho\sigma$. This equation has been used to correlate the effects of polar substituents on the rates and equilibria of many reactions of aromatic compounds. Each substituent is characterized by a value, σ, a measure of its electron-withdrawing or donating property, a positive value indicating electron-withdrawal, negative value indicating electron-donation, and higher absolute values corresponding to greater effects. Each reaction is characterized by a value, ρ, that for the ionization of benzoic acids having value 1, positive values indicating processes increased by electron withdrawal, and negative values for processes increased by electron-donation. In brief, (i) electron-attracting substituents increase binding and electron donation decreases binding in a series of eleven compounds, from $K_{i(com)} = 0.46$ for 4-nitroacetanilide to $K_{i(com)} = 50$ for phenol; (ii) dimethylamino and tert-butyl substituents increase binding, apparently by interaction at the trimethyl subsite; (iii) this interaction increases binding synergistically in meta-substituted phenols; and (iv) the binding effects due to cationic substituents in PTA and TMAP are accounted for by their σ values, just as are those of nitro, acetyl, etc., with no additional effect such as interaction with an anionic charge; the binding of aromatic substituents of inhibitors and substrates is attributed to charge-transfer interaction with aromatic amino acid side

chains of the active site; the trimethyl and aryl binding areas are contiguous, forming a single domain of the active site (24).

It is noteworthy that nitrobenzene, $K_{i(com)}$ 0.6 mM, and acetophenone, $K_{i(com)}$ 4 mM, accelerate hydrolysis of the small substrate, ethyl acetate, much as small cations do (25). Also, nitrobenzene has little effect on inactivation by BrPin.

2. Pyridines

We have extended study of aromatic reversible inhibitors to pyridine derivatives, and results will be described in a later section. It is noteworthy that dimethylaminopyridines (DMAPy), bearing no permanent positive charge, may bind as strongly as N-methylpyridinium ion (NMPy), and thus may lead to strongly binding reagents with superior permeating properties.

3. Irreversible Inhibitors

Action of irreversible aromatic inhibitors was described briefly in the Midterm Report, (1). Inactivation by styrene epoxide is retarded by 5-trimethyammonio-2-pentanone (TAP). Inactivation by MBS is retarded by TAP but not by TeMA. Phenacyl bromide inactivated as effectively as styrene epoxide and may be a more readily accessible radioactive labeling agent. Further studies of PhABr and MBS are reported under Results.

D. X-Ray Diffraction

As this contract drew to a close, an X-Ray diffraction study of AcChE from T. californica was published (26). The active site is a deep cleft in which the ammonium substituent is bound not to an "anionic" site, but to aromatic residues that line the site. This confirms our general view of the active site.

III. Rationale for the Current Study

(A) BrPin has properties of an active-site-directed inactivator, being equally effective against cationic, uncharged and aromatic substrates, and its own action is retarded by substrate-related reversible inhibitors (17). [^{14}C]BrPin is being used as a radioactive label to identify amino acid residue(s) in the active sites of AcChEs from T. nobiliana and Electrophorus. The enzymes are isolated from the electric organs and purified and inactivated by [^{14}C]BrPin (in the presence and absence of reversible inhibitors) and cleaved into fragments which are characterized and sequenced. Enzyme content is determined fluorometrically, allowing calculation of the number of ^{14}C introduced per enzyme unit inactivated by [^{14}C]BrPin.

(B) Noncompetitive inhibition is studied for evidence for formation of ES·I′ and E·S·S′ complexes.

(C) Study of substituted benzenes and pyridines indicates that they may be effective reversible inhibitors in the absence of permanent positive charge. This may impart superior permeating properties, combined in the pyridines with increased water solubility. Preparation of benzene- and pyridine-derived labeling reagents is being studied with a view to identifying other residues in the active site. Preparation of uncharged pyridine-derived reactivating reagents will be explored.

(D) Information about mechanism of inactivation by BrPin may be obtained by study of effects of pH on the inactivation.

IV. Experimental Methods

A. Chemicals

Sources of the chemicals not described in the Midterm Report (1) were as follows: Dithiothreitol (DTT), tetrapropylammonium chloride, tripropylammonium chloride, phenyltrimethylammonium iodide, 3-tert-butylphenol, benzenesulfonyl chloride (BSCl), 1,1-trimethylene-bis-(4-hydroxyiminomethyl) pyridinium bromide (TMB-4), 2,2′-dipyridyldisulfide (PDS), iodoacetic acid (IAc), iodoacetamide (IAm), trifluoroacetic acid (TFA), phenylmercuric chloride (PMC), n-methyldiethanolamine, phenacyl bromide (PhABr), mp 49-50°C, from ethanol, and 4-dimethylaminobenzaldehyde were from Aldrich Chemical Co. (Milwaukee, WI); 5-dimethylamino-2-pentanone was from Sapon Laboratories (Bloomsbury, NJ); 4-nitropyridine-N-oxide and methyl benzenesulfonate (MBS) were from Pfaltz and Bauer (Waterbury, CT); N-methylacridinium iodide was from Molecular Probes Inc. (Eugene, OR); diisopropyl fluorophosphate (DFP), human serum butyrylcholinesterase and horse serum butyrylcholinesterase, human erythrocyte AcChE, AcChE from Electrophorus, somatostatin, bradykinin, bovine serum albumin, trypsin (type XIII), acetylthiocholine (AcSCh), propidium iodide, S-carbamyl-L-cysteine, p-nitrophenylphosphoryl choline, tubocurarine chloride, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), were from Sigma Chemical Co. (St. Louis, MO); trimethylammoniodibromobimane, and bromobimane were from Calbiochem (San Diego, CA); [^{3}H]DFP, [^{14}C]-acetyl chloride, [^{3}H]BH_4^- and ENHANCE were from New England Nuclear (Boston, MA); NCS tissue solubilizer and biodegradable scintillant cocktail was from Amersham (Arlington Heights, IL); acrylamide, N,N′-methylene-bis-acrylamide and ammonium persulfate were from BioRad (Rockville Centre, NY); 2,6-di-tert-butylpyridine was from Fluka Biochemika (Buchs, Switzerland); decamethonium bromide (DeMe) was from ICN pharmaceutical (Irvine, CA); 3-dimethylaminoacetophenone was from Spectrum Medical Industries (Giardena, CA); 2-dimethylaminomethyl-3-hydroxypyridine was from Chem. Service (West Chester, PA); Sephadex G-50 was from Pharmacy Biotechnology, (Piscataway, NJ).

B. Syntheses

Procedures reported in the Midterm Report (1) are not repeated here. They included syntheses of 2-oxyrilpyridine, pyridine-N-oxide, 3-dimethyl-aminopyridine, N-methylpyridinium iodide, 3-bromoacetylpyridine, 4-nitropyridine, 3-trimethylammonioacetophenone, 2-N,N-dimethylaminoethyl acetate, N,N-dimethyl-N′-acetylethylenediamine, 3-phenyl-3-trifluoromethyldiazirine, and 3-(3′-iodophenyl-3-trifluoromethyldiazirine. The availability of TAP, DMBAc, 2-methylsulfonylethyl acetate, n-butyl acetate, N,N-dimethylamino-2-ethoxyethane and N,N,N-trimethylammonio-2-ethoxyethane iodide from previous studies was also reported there.

Synthesized compounds had physical properties in accord with literature values where available. Analytical data are reported for newly synthesized compounds.

Pivalophenone (1,1,1-trimethylacetophenone) was prepared by slow addition of benzoic acid (8 mmol) in toluene to 26 mmol of tert-butyllithium, 1.7 M in pentane. The mixture was stirred overnight, poured into water, extracted with ether and distilled, bp 33-34°C/0 15 mm.

2-(Methylammonio)ethyl acetate hydrochloride (MAAc) was prepared from 2(methylamino)ethanol and excess acetic acid saturated with hydrogen chloride at room temperature, mp 120°C from acetone. 2-(Diethylamino)ethyl acetate was prepared from the alcohol and acetyl chloride in ether followed by treatment with base and extraction with ether, bp 69-71°C/5 mm. This was heated at 80°C in excess n-butyl iodide for 8 hr under nitrogen, followed by dilution and washing with ether, and crystallization from ethanol-ether, leading to 2-(N,N-diethyl-N-butylammonio)ethyl acetate iodide (DEBAAc), mp, 99-100°C. Sources of other substrates examined in the study of noncompetitive inhibition have been described (20).

5-N,N-Diethyl-N-n-butylammonio-2-pentanone (DEBAP) iodide was prepared from 5-diethylamino-2-pentanone and n-butyl iodide, mp 105-106°C. N,N-Diethyl-N-neopentylamine was prepared from neopentylamine, formaldehyde and formic acid, bp 65-67°C. N,N,N-Trimethyl-N-neohexylammonium iodide was prepared from neohexylamine and methyl iodide. Dimethylaminoethylethyl ether was prepared from β-bromoethyl ethyl ether and aqueous dimethylamine. The source of other reversible inhibitors has been described (20).

Methylcarbamyl choline: Dimethylaminoethanol (0.05 mol) was refluxed with 0.07 mol methylisocyanate in toluene for 17 hr and methylcarbamyl dimethylaminoethanol was obtained, bp 98°C/5 mm; 1.5 g of this was treated with 1.7 g of methyl iodide in acetone

at room temperature, leading to 2.6 g of methylcarbamyl choline iodide, mp 175-176°C.

Triethyleneglycol di-tert-butyl ether was prepared by conversion of the glycol to its di-p-toluenesulfonate, followed by treatment with potassium tert-butoxide in tert-butyl alcohol, bp 69°C/0.03 mm.

The dipivaloate ester hydrochloride of N-methyldiethanolamine was prepared from 0.1 mol of N-methyldiethanolamine and 0.25 mol pivaloyl chloride in chloroform under nitrogen, mp 143°C, from acetone, Anal. calc'd for $C_{15}H_{30}NO_4Cl$: C, 55.63; H, 9.39; N, 4.32. Found: C, 55.95; H, 9.32; N, 4.32.

The tert-butylacetate ester hydrochloride of N-methyldiethanolamine was prepared from 0.070 mol N-methyldiethanolamine and 0.14 mol tert-butylacetylchloride, mp 56°C, from acetone. Anal. calc'd. for $C_{17}H_{34}NO_4Cl$: C, 58.02; H, 9.74; N, 3.98. Found: C, 58.31; H, 9.78; N, 3.98.

The diacetate hydrochloride of N-methyldiethanolamine was prepared from 0.1 mol of N-methyldiethanolamine and 0.3 mol of acetyl chloride in chloroform, mp 101°C, from acetone.

Microscale synthesis of PhABr in preparation for synthesis of radioactive material was successful: 120 μmol acetophenone treated with 108 μmol Br_2 in ether in the presence of HCl, followed by water wash, led to PhABr, 80% by HPLC, which inactivated AcChE similarly to purchased material.

Microscale reaction of MBS: 100 μmol benzenesulfonyl chloride (BSCl), and 130 μmol methanol in ether left residual BSCl. BSCl was found to inactivate AcChE from Electrophorus more than 20 times as rapidly as MBS, and residual BSCl must be removed. Reaction in the presence of 100 μmol 2,6-di-tert-butylpyridine failed. Washing with 100 μmol diethanolamine in water or reaction in the presence of the base left residual BSCl. Reaction with sodium methoxide in dimethylformamide failed. Reaction of 100 μmol BSCl and 130 μmol methanol in 250 μL ether, 21 hr, 25°C, then stirred 2 hr with 110 μmol NaOH in 10 μL H_2O, then washed with 2 x 100 μL H_2O, led to 72% MBS, free of BSCl.

S-Pinacolonylcysteine hydrobromide: Cysteine (12.1 g, 0.10 mol) suspended in 100 mL methanol was treated with 48% aqueous HBr (16.9 g, 0.10 mol) and concentrated. The cysteine hydrobromide was washed with ether and dried in vacuum, 20 g., 100% yield, mp 123-125°C. BrPin (3.0 mL, 20 mmol) was added to cysteine hydrobromide (4.0 g, 20 mmol) in 20 mL methanol, and the solution was stirred overnight under nitrogen, concentrated, triturated and warmed with methanol and ether, cooled, filtered, washed with ether and dried, 1.8 g, 30% yield, dec. > 170°C.

Anal. Calc'd for $C_9H_{18}NO_3SBr$: C, 36.01; H, 6.04; N, 4.67. Found, C, 36.49; H, 5.68; N, 4.98. The compound is insoluble in $CDCl_3$, slightly soluble in D_2O, soluble in DMSO-d_6, in which, however, it decomposes rapidly, the solution becoming deep orange; it is soluble in CH_3OH-d_4, in which it decomposes more slowly. In the NMR, the tert-butyl hydrogens are clearly observed in CH_3OH-d_4, as a sharp singlet; the pinacolonyl-CH_2 was not observed, and part of the cysteine-CH_2 quartet, split into a doublet by the adjacent C-H, was overlapped by residual solvent-H. A ratio of 9:2:1 is observed in D_2O for the three sets of protons; the pinacolonyl-CH_2 may be masked by residual -OH. All seven peaks for S-pinacolonylcysteine were observed in C-13 NMR in D_2O.

S-Pinacolonycysteine: A solution of 0.034 mL BrPin in 25 mL H_2O (10 mM) was brought to pH 7.5 by 0.4 mL of pH 8.2 phosphate buffer, and 30 mg of cysteine was added. This was intended to be an equivalent of cysteine, but subsequent analysis with 2-PDS indicated 62% -SH, the remainder largely cystine. Analysis shortly after mixing indicated essentially complete disappearance of -SH, apparently by addition to the pinacolonyl carbonyl. The pH dropped rapidly, to 3.5 in 10 min, 2.7 in 1 hr, and was brought back to pH 6 with NaOH as the alkylation proceeded. The reaction was followed by HPLC, C-18 column, H_2O + 0.1% TFA to 1:1 H_2O:CH_3CN + 0.075% TFA, and compared with elutions of 10 mM standards of cysteine and BrPin. The product eluted after cysteine and before BrPin. The reaction was essentially complete after 1 hr, the large product peak not changing on further standing. A portion of the reaction solution, 12 mL, was taken to dryness and the pinacolonylcysteine was isolated by repeated HPLC elutions of small portions. HPLC of the separated product showed no other materials present. This product was subjected to the sequencing reagents and conditions. Another portion of the reaction solution, 5 mL, was brought to pH 7.1 and allowed to stand and aliquots were examined over a period of several days. The solution gradually became yellow and side bands appeared about the product peak as the compound decomposed.

C. Isolation of Acetylcholinesterase

(1) From T. nobiliana (27). The tryptic digestion protocol of Taylor et al. [27] in conjunction with affinity chromatography was used to isolate 11S AcChE from T. nobiliana. The affinity ligand, N-(6-aminohexanoyl-m-aminophenyl)trimethylammonium bromide hydrobromide, was prepared from 6-aminohexanoic acid and N,N-dimethyl-1,3-phenylenediamine (29, 30). It was coupled to 6-aminohexanoic acid-Sepharose B with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and the resin was stored in buffer (0.1 M NaCl, 40 mM $MgCl_2$, 10 mM $NaHCO_3$ (pH 8)) containing 0.1% sodium azide. Frozen electric organ was obtained from Biofish Associates (Gloucester, MA). The isolation procedures (28) were carried out at 4°C, except for the tryptic digestion. Tissue, 500 g, was homogenized in 1500 mL of the pH 8.0 buffer and centrifuged at

9000 x g. The pellet was resuspended (250 mL), treated with trypsin (5 μg/mL) at 37°C for 10 min, and then with trypsin inhibitor (10 μg/mL). The suspension was centrifuged at 27,000 x g, and the supernatant was assayed and applied to the affinity column (1.5 x 20 cm) and washed. Enzyme was eluted with the buffer, containing 1 mM decamethonium. Fractions high in activity as determined by Ellman assay (31) were pooled (about 30 mL), freed of decamethonium by CM Sephadex (1.5 x 15 cm), pooled again, concentrated, diluted and reconcentrated (4-5 mL) with an Amicon ultrafiltration apparatus (30 kDa membrane cutoff), and stored as 0.2 mL aliquots at -70°C. Aliquots were thawed and used directly and the preparations retained full activity over several months of storage.

(2) Two eels were purchased from World Wide Scientific Animals, Opopka, Florida. They were cooled, decapitated and skinned, and the electric organs separated, 800 g, and stored at -70°C. A portion, 130 g, was homogenized in pH 7.8 buffer and centrifuged. The pellet was resuspended and treated with 35 mg trypsin at 37°C and assayed after stated periods of incubation. After affinity chromatography, 3.1 mg of enzyme in 3.9 mg protein was isolated and stored at -70°C in 30 0.2 mL aliquots.

D. Assays of Acetylcholinesterase

The Ellman assay solution contained 0.05 M (pH 7.8) phosphate buffer, 0.5 mM AcSCh, 0.16 mM DTNB and 0.1-1 nM enzyme. Initial increase in absorption was recorded at 410 nm, $\epsilon = 1.36 \cdot 10^4\ M^{-1} \cdot cm^{-1}$, against a reference cell containing all components except enzyme. For enzyme concentrations, rates were corrected to V_{max} from data obtained in determination of K_m.

Active site concentration of AcChE was determined by titration with the carbamoylating agent M7CQ (32, 33). Fluorescence was measured on a Hitachi Perkin Elmer MPF-4 spectrofluorimeter; excitation of M7HQ was at 400 nm (slit width 10 nm), emission maximum at 510 nm (slit width 12 nm), lit, 505 nm (32), 510 nm (33). Corrections were applied for enzyme scatter and small emission from M7CQ. Protein was determined by the Lowry method (34) with Bicinchonic Assay Kit, Sigma Chemical Co., and bovine serum albumin and γ-globulin standards.

E. Gel Electrophoresis (35)

Slab gels were 13 x 16 x 0.15 cm, 8% acrylamide, 0.3% N,N'-methylenebis(acrylamide) (pH 8.0), 0.1% SDS; stacking gel was 4% acrylamide and 0.15% of the bisacrylamide. The electrode buffer was 0.05 M Tris, 0.38 M glycine, 0.1% SDS, 0.1 mM sodium thioglycolate (pH 8.3). Concentrated Torpedo AcChE (0.2 mL) was diluted with pH 7 phosphate buffer and treated with [^{14}C]BrPin, DFP, etc., as desired. The solutions were made 1% in 2-mercaptoethanol and 10% in sample buffer (20% sucrose, 10% SDS,

0.12 M Tris (pH 6.8), 0.025% bromophenol blue, 1% 2-mercaptoethanol, 25% glycerol) and heated at 100°C for 5 min, and aliquots, generally 0.10 mL, were applied to the gel along with molecular mass markers: carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase b (97.4 kDa), β-galactosidase (116 kDa) and myosin (205 kDa). Electrophoresis was run for about 6 hr at 100 V. Gels were fixed for 20 min in 5:4:1 methanol/water/acetic acid, stained for 3 hr in 0.25% Coomassie Blue in 5:5:1 methanol/water/acetic acid, and destained in 2.5:6.5:1 methanol/water/acetic acid.

F. Counting and Autoradiography

(1) Counting of gel. Sections of the lanes of slab gel were placed in scintillation vials containing 0.9 mL of NCS (Nuclear Chicago Solubilizer) tissue solubilizer and 0.1 mL of H_2O, heated at 50°C for 2 hr and cooled. Econofluor, 10 mL, was added, and counts were obtained, generally for 5 min per vial, on a Beckman LS-100C liquid scintillation system.

(2) Counting of incubations. Enzyme concentrate (1.5 mL) was diluted to 7.0 mL in pH 7 buffer and an aliquot was partially inactivated, treated with azide and dialyzed at room temperature for 48 hr against 1L of buffer, changed after 24 hr, and counted in 10 mL of biodegradable scintillation cocktail.

(3) The slab gel was immersed in ENHANCE for 1 hr, then in 1% glycerol/5% acetic acid for 1 hr, dried on a Bio-Rad dryer (Model 224) at 60°C, for 2-3 hr and placed in flashed (36) X-OMAT AR film at -70°C.

G. Kinetic Procedures

1. In evaluation of competitive and noncompetitive components of reversible inhibition of hydrolysis of substrates of varied reactivity, kinetic studies were carried out titrimetrically at pH 7.8, 25°C, in 0.18 M NaCl under argon with Radiometer Titrator TTT 80, Autoburette ABU80, pH meter PHM82 and Kipp and Zonen BD 40 chart recorder. AcChE (Electrophorus EC 3.1.1.7, V-S lyophilized, Sigma), 1000 units, was dissolved in 20 mL of 0.18 M NaCl and stored at 4°C. An aliquot was assayed before each substrate inhibitor by hydrolysis of AcCh with k_{cat} = 1.6×10^4 s^{-1} (7). Rates of hydrolysis were generally determined at three to six concentrations of inhibitor and substrate. Least-squares analysis of inverse rate vs. inverse substrate concentration led to slope and intercept at each concentration of inhibitor, correlations 0.98-0.99+. Slopes and intercepts of secondary plots of slopes of the 1/V vs. 1/S data against inhibitor concentrations were calculated and ratios of these secondary intercepts to slopes gave values of competitive inhibition constants, $K_{i(com)}$. The values of $K_{i(com)}$ are averages for each inhibitor of the values obtained with all the substrates

studied with it. Similar treatment of secondary intercepts and slopes of intercepts of 1/V vs. 1/S data gave values of noncompetitive inhibition constants, $K_{i(nonc)}$, with each substrate. Noncompetitive inhibition as used here refers to the observation that the V_{max} values observed from extrapolation of the 1/V vs. 1/S data decrease with increasing concentration of reversible inhibitor (37).

2. For reversible inhibition by PhABr, stock solutions of PhABr in HPLC grade CH_3CN and of AcCh in 0.18 M NaCl were prepared. Appropriate aliquots were combined and added to AcChE in 0.18 M NaCl, and hydrolysis was followed titrimetrically.

Inactivation of AcChE from Electrophorus by PhABr, generally 1 x K_i, alone and in the presence of reversible inhibitors, generally 10 x K_i, was followed over a period of 6 hr, and the gradual loss in activity was observed at time intervals.

H. 1-Bromo[2-^{14}C]Pinacolone ([^{14}C]BrPin) (27)

[1-^{14}C]-Acetyl chloride, 1 mCi, 60 μmol, (16.7 mCi/mmol) prepared from freshly distilled 57 mCi/mmol material by dilution with acetyl chloride, was obtained from New England Nuclear. The acetyl chloride was transferred under dried nitrogen by syringe in three 50 μL portions of ether, which had been freshly distilled from sodium benzophenone ketyl, to a 300-μL nitrogen flushed Reacti-Vial (Pierce Chemical, Rockford, IL) fitted with a Teflon-lined silicone septum and containing 3 mg of anhydrous Cu_2Cl_2 and a spin bar. The Reacti-Vial was cooled in ice and 30 μL of 2 M tert-butyl-magnesium chloride (60 μmol) was added by syringe, forming a white precipitate, and the mixture was stirred for 30 min. Bromine, 3 μL (59 μmol), and 50 μL of dry ether were added by syringe, and the mixture was stirred at room temperature until the orange color was replaced by green, about 2 hr. The mixture was washed in the Reacti-Vial with two 30 μL portions of water, with rapid spinning, and the aqueous layers were removed. The colorless ether solution was analyzed by gas-liquid chromatography and found to contain 21 μmol of [^{14}C]BrPin (35% yield), and 10 μmol of 1,1-[2-[^{14}C]-dibromopinacolone, [^{14}C]Br_2Pin (17% yield). It was stored as 0.12 M [^{14}C]BrPin in ether at -5°C in the Reacti-Vial fitted with a solid cap lined with a rubber disc and gold foil. In a parallel synthesis, carried out simultaneously with nonradioactive acetyl chloride, the yield of BrPin was 50% and the ratios of pinacolone, BrPin and Br_2Pin were 0.6:1.0:0.4.

I. Inhibition by 1-Bromopinacolone

(1) Inactivation of Torpedo AcChE by BrPin and by [^{14}C]BrPin was studied in 0.1 M phosphate (pH 7.0) in the presence and absence of TAP or decamethonium, by Ellman assay of residual activity after periods of incubation. Enzyme

preinactivated by DFP or [^{3}H]DFP was allowed to stand overnight at 4°C before treatment with [^{14}C]BrPin. Reaction with BrPin was terminated by 0.1 M sodium azide; thus, incubation of 0.01 μM AcChE with 2 mM BrPin led to 42% inactivation in 80 min; after addition of azide, activity fell about 5% over the next 85 min.

(2) Values of K_i were determined by Lineweaver-Burk treatment of 1/V and 1/S data (37). Ratios of secondary intercept and slope of slope vs. inhibitor concentration data led to values of $K_{i(com)}$. Inhibition of Torpedo AcChE was examined titrimetrically at four concentrations of AcCh, 0.06-0.30 mM, and of BrPin, 0.105-0.525 mM. Secondary intercepts and slopes were 35.3 ± 9.2 s and (1.75 ± 0.33) · 10^5 M^{-1} · s, respectively. Inhibition of Torpedo AcChE by 0.0, 0.05 and 0.10 mM TAP was examined by the Ellman procedure at six concentrations of AcSCh, 0.04-0.50 mM. Secondary intercepts and slopes were (1.83 ± 0.01) · 10^3 s and (2.42 ± 0.01) · 10^7 M^{-1} · s, respectively. Pinacolone and Br_2Pin were examined titrimetrically on Electrophorus AcChE at six concentrations of AcCh, 0.10-0.60 mM, at four concentrations of pinacolone, 5-20 mM, and at 0.25 mM Br_2Pin. Secondary intercepts and slopes were 92.9 ± 7.7 s and (1.68 ± 0.06) 10^4 M^{-1} s, respectively.

J. Isolation of Labeled Peptides

(1) Inactivation by nonradioactive BrPin: Solutions of enzyme (0.8 mg of protein, 7.3 μM active enzyme in 1.6 mL of phosphate buffer, pH 7.0, I = 0.18) containing (i) enzyme only, (ii) enzyme plus 1.0 mM BrPin, and (iii) enzyme, 1.0 mM BrPin and 14 mM TAP, were incubated 5 hr at 25°C and assayed at intervals. Aliquots, 2.5 μL, were diluted in 1.0 mL of buffer, and 0.05 mL portions were assayed in 2.0 mL of the Ellman solution. To terminate action of BrPin, the incubations were made 0.1 M in sodium azide and kept 20 min at room temperature. Samples were dialyzed against 0.1 M tris buffer, pH 8.0, then adjusted to 4.5 M guanidinium chloride and 4.0 mM DTT and incubated 1 hr at 50°C under nitrogen. After cooling the solution was made 9.0 mM in IAc, kept 1 hr at room temperature in the dark, and then dialyzed against 50 mM NH_4HCO_3, pH 8, overnight. The solutions were treated with trypsin (40 μg, 5% w/w) at 37° for 24 hr. Aliquots equivalent to 40 μg of initial protein, taken before and after digestion with trypsin, were subjected to gel electrophoresis, and the major parts of the digests were freeze dried. The samples were reconstituted in 150 μL of H_2O:0.1% TFA, and 100 μL portions were chromatographed on a Rainin Rabbit-HP HPLC system using a C-18 Column, Dynamax-300Å, 4.6 x 250 mm. The peptides were eluted by a linear gradient, H_2O:0.1% TFA to 1:1 CH_3CN/H_2O:0.075% TFA, flow rate 0.5 mL/min. Fractions (0.5 mL) were collected with continuous recording of absorbance at 219 nm (HM Holochrome Detector). Subsequent increase of gradient from 50% to 80% CH_3CN/H_2O, 0.075% TFA led to no additional elution of peptide or radioactivity.

(2) Inactivation by [^{14}C]BrPin

a. Solutions of enzyme (1.25 mg of protein, 3.1 μM active enzyme in 5.6 mL of phosphate buffer, pH 7.0) containing (i) enzyme only, (ii) enzyme made 0.6 mM in [^{14}C]BrPin, and (iii) enzyme, 0.6 mM [^{14}C]BrPin and 15 mM TAP, each brought in the process to 7.6 mL total volume, were incubated 8.5 hr at 25°C and assayed and treated as in (1) with initial dialysis for 48 hr and digestion with 63 μg trypsin (5% w/w). The samples were reconstituted in 120 μL of H_2O:0.1% TFA, and 100 μL of each were subjected to HPLC as in (1). Aliquots (0.025 mL) from each 0.5 mL HPLC fraction of (ii) and (iii) were counted on a Beckman LS-100 C counter.

b. Solutions of enzyme (2.0 mg of protein, 5.5 μM active enzyme in 4.1 mL of buffer) containing (i) enzyme only, (ii) enzyme made 1.2 mM in [^{14}C]BrPin, and (iii) enzyme, 1.2 mM [^{14}C]BrPin and 15 mM TAP were incubated 24 hr at 25°C and assayed and treated as in (2)a., with digestion by 40 μg trypsin (2% w/w). The samples were reconstituted in 220 μL of H_2O:0.1% TFA, and 100 μL of each was subjected to HPLC, and aliquots (0.02 mL) from each HPLC fraction of (ii) and (iii) were counted.

K. Amino Acid Sequence Analysis

The HPLC fractions which were affected by the reaction with BrPin or [^{14}C]BrPin and corresponding fractions in non-inactivated samples were freeze-dried, stored at -20°C, and then reconstituted in 200 μL of 30% CH_3CN:H_2O for sequence analysis (38) in an Applied Bioystems Model 470 A gas phase protein sequencer (39); 40% of the released phenylthiohydantoin (PTH)-amino acid was separated on an on-line Model 120A PTH amino acid analyzer, and approximately 40% was used for determination of radioactivity. Cycle yields of PTH amino acids were quantified from HPLC peak heights. To quantify yields of the Ala-222 peptide, initial yield (I_o) and repetitive yield (R) were calculated by nonlinear least squares regression of observed release (M) at each cycle (n): $M = I_oR^n$. For samples where the Ala-222 peptide was the clear primary peptide, only PTH, Cys, Ser, and Trp were omitted from the analysis. For samples, where the Ala-222 was present among other peptides (i.e., fraction 76), comparison of sample from BrPin-treated enzyme with untreated controls identified amino acids in six cycles that were unique to the Ala-222 peptide: Ala in cycles 1 and 13, Ile in cycle 2, Gly in cycle 6, and Pro in cycles 8 and 11. Only these residues were used to estimate initial and repetitive yields. To further identify the amino acid reacting with [^{14}C]BrPin, ortho-phthalaldehyde (OPA) was used to block primary amine amino termini exposed at a particular cycle of Edman degradation while leaving sequences with NH_2-terminal proline susceptible to subsequent Edman degradation (40); the protocol used was that of Middleton and Cohen (41): The sequencer was stopped briefly at

the desired cycle, and the filter was treated for 20 sec with 12.5% aqueous trimethylamine and then dried. The filter cartridge was disassembled, and 30 μL of OPA reagent (2 mg OPA/mL in CH_3CN/0.5% mercaptoethanol) was added to the filter. The cartridge was reassembled and the filter was treated for 10 min with 12.5% aqueous trimethylamine, dried with argon and rinsed with ethyl acetate before normal sequencing was resumed.

L. Inhibition by Sulfhydryl Reagents

1. Reversible inhibition by 2-PDS: Reversible inhibition by 0.023, 0.046, 0.084, and 0.172 mM 2-PDS of hydrolysis of 0.075-0.40 AcCh by 0.03 nM AcChE from T. nobiliana, pH 7.8, 0.18 M NaCl, was examined titrimetrically (20). Reversible inhibition by 0.30, 0.60, and 0.90 mM 2-PDS of hydrolysis of 0.1-0.6 mM AcCh by 0.23 nM AcChE, Sigma Electrophorus, pH 7.8, 0.18 M NaCl, was examined similarly.

2. Sulfhydryl reagents as inactivators of AcChE from T. nobiliana; 2-PDS, DTNB, IAm, IAc: Incubations, 0.5 mL, in phosphate buffer, pH 7.8, I = 0.2, containing (i) AcChE (ii) AcChE and inactivator, (iii) AcChE, inactivator and TAP, were examined for residual activity. After periods of standing at 22°C, aliquots, 0.05 or 0.025 mL, were diluted 160-fold, and residual activity was monitored by Ellman assay.

In inactivation by varying concentrations of 2-PDS, aliquots (0.05 mL) of 0.5 mL incubations of 17 nM enzyme with 2 and 4 μM 2-PDS, and 45 nM enzyme with 10, 20, 40, 80, and 180 μM 2-PDS were analyzed for residual activity at intervals over a period of 1.6 hr. In inactivation of 5.3-9.6 nM AcChE by a range of concentration of BrPin, remaining activity was followed during incubation at pH 7.0 over periods of 1.7-3.0 hr with 0.02-2.0 mM BrPin. In protection by TAP against inactivation by 2-PDS, aliquots of incubations of 100 nM enzyme and 56 μM 2-PDS containing 0, 0.5, 1.0, and 3.0 mM TAP were assayed for residual activity over a period of 1.2 hr.

3. Exclusion of M7CQ from inactivated AcChE. Aliquots (0.5 mL) of 0.25 μM enzyme in pH 7.8 phosphate buffer with (i) 0.92 mM BrPin and (ii) 0.14 mM 2-PDS were allowed to stand at 4°C for 70 hr. They were then analyzed for remaining activity by Ellman assay, and examined for reaction with M7CQ (33, 27).

M. Other Cholinesterases

Human erythrocyte AcChE, 0.8 mg, was suspended in 1 mL of phosphate buffer and centrifuged. The supernatant was divided into three portions, 0.8 mM BrPin was added to the second portion, and 0.18 mM 2-PDS was added to the third portion; 0.05 mL aliquots were assayed by the Ellman method. Horse serum cholinesterase (500 units, 0.83 mg of protein) was suspended in

4.0 mL H_2O and 0.2 mL phosphate buffer, pH 7.8, I = 0.2. Aliquots (0.3 mL) were taken: (i) diluted with 0.2 mL buffer; (ii) made 1.0 mM in BrPin, final volume 0.5 mL, and (iii) made 0.2 mM in 2-PDS, final volume 0.5 mL; 0.05 mL aliquots of each were assayed by the Ellman method. Human serum cholinesterase, 10 units, 2.2 mg of protein, was suspended and treated in the same way with BrPin and 2-PDS. Assays were carried out after periods of incubation. Reversible inhibition by BrPin of hydrolysis of 0.056-0.11 mM AcSCh by human BuChE was examined by the Ellman procedure. Concentrations of BrPin (mM) and linear regression slopes (s) and intercepts ($M^{-1}s$) of 1/V vs. 1/S plots were: 0, 2.8×10^2, 8.4×10^6; 0.075, 3.1×10^2, 10.0×10^6; 0.150, 4.0×10^2, 9.6×10^6; 0.375, 5.0×10^2, 11.0×10^6.

N. Reduction by $[^3H]BH_4^-$

Enzyme aliquots treated with PhABr or BrPin in the absence and presence of TAP and untreated controls were treated with azide, dialyzed, denatured with guanidinium ion, reduced by DTT, alkylated with IAc, and then treated with $[^3H]BH_4^-$. After treatment they were acidified, freed of $^3H_2$ in a stream of N_2, dialyzed, and counted.

V. Results

A. Reaction of [^{14}C]BrPin with AcChE from T. Nobiliana; Effects of TAP, DeMe and DFP

1. Synthesis of [^{14}C]BrPin. [^{14}C]BrPin was prepared on a 60 μmol scale by treatment of [1-^{14}C]-acetyl chloride with tert-butylmagnesium chloride in ether, followed by reaction with bromine, eqs. 2, 3 (27).

$$CH_3{}^{14}COCl + (CH_3)_3CMgCl \xrightarrow{Cu_2Cl_2} CH_3{}^{14}COC(CH_3)_3 + MgCl_2 \quad (2)$$

$$\xrightarrow{Br_2} CH_3{}^{14}COC(CH_3)_3 + BrCH_2{}^{14}COC(CH_3)_3 + Br_2CH{}^{14}COC(CH_3)_3 \quad (3)$$

[1-^{14}C]-Acetyl chloride deteriorates on storage and must be obtained freshly prepared, Cu_2Cl_2 catalyst is essential for a high yield of pinacolone, a small excess of acetyl chloride leads to successful bromination, and thorough washing out of cupric ion and any residual bromine, which inactivate AcChE instantly, is necessary. The reaction sequence leads to pinacolone, BrPin and Br_2Pin, in a ratio of about 1:2:1, in an ether solution, which is used directly. BrPin has $K_{i(com)}$ 0.20 mM with AcChE from T. nobiliana, $K_{i(com)}$ 0.18 mM with AcChE from Electrophorus; pinacolone has $K_{i(com)}$ 5.5 mM, while Br_2Pin, a mixed inhibitor, K_i 0.8 mM, does not inactivate AcChE under the conditions of our studies.

2. AcChE from Torpedo nobiliana (27). AcChE was extracted from T. nobiliana electric organ by limited tryptic digestion (28). Based upon the Ellman assay, about 25% of AcChE activity was released by tryptic digestion; about 55% of solubilized activity was recovered after chromatography, removal of decamethonium, and concentration. Final yield of protein was 18 mg/kg of tissue, with abs $_{280}^{1\%}$ = 22. Specific activities were 2400 μmol AcSCh hydrolyzed/min and 3400 μmol AcCh hydrolyzed/min/mg of protein. Analysis of the polypeptide composition by SDS-PAGE showed a major band at about 70 kDa and minor bands at higher molecular masses. Enzyme samples, treated separately with [^{3}H]DFP and [^{14}C]BrPin, followed by SDS-PAGE and autoradiography, showed major introduction of labels at 70 kDa in both cases. The minor higher molecular weight bands also showed incorporation of radioactivity.

Active site concentration was determined by titration with the carbamylating agent M7CQ. The burst release of the fluorescent product, M7HQ, was proportional to the volume of enzyme solution assayed, and established that a preparation containing 1.00 ± 0.07 mg/mL protein contained 10.2 ± 0.2 μM active site. Comparison of active site and protein concentration indicated that at least 70% of the protein was present as active catalytic subunit, based upon a catalytic subunit mass of 70 kDa. Slow release of M7HQ after the initial burst leads to the

decarbamylation rate constant, 1.5×10^{-4} s^{-1}. The rate of carbamylation studied at low concentration of M7CQ leads to an apparent first-order rate constant, 3.8×10^{-3} s^{-1}, which approximates to $k_{+2}[I]/K_i$, where k_{+2} is the carbamylation constant in the enzyme-inhibitor complex, and k_{+2}/K_i is the second-order carbamylation rate constant. With $k_{+2}/K_i = 8.0 \times 10^3$ M^{-1} s^{-1}, and with $K_i \sim 10$ μM (33), $k_{+2} = 0.08$ s^{-1}, similar to the value for Electrophorus AcChE (33).

Lineweaver-Burk analysis of rates of hydrolysis of 0.017-0.25 mM AcSCh by 0.1-1.2 nM AcChE from T. nobiliana, at pH 7.8, determined by the Ellman method, leads to $K_m = 0.055 \pm 0.008$ mM as compared with 0.077 mM for AcChE from T. marmorata (42), and $k_{cat} = (4.0 \pm 0.04) \times 10^3$ s^{-1} as compared with 5.5×10^3 s^{-1}. Hydrolysis by 0.18 nM AcChE of 0.056 - 0.296 mM AcCh, examined titrimetrically, led to $k_{cat} = (5.6 \pm 0.2) \times 10^3$ s^{-1}, $K_m = 0.051 \pm 0.003$ mM.

3. Reaction of [^{14}C]BrPin with Torpedo AcChE; Effects of TAP, decamethonium and DFP. The solution of 1 mM [^{14}C]BrPin, containing in addition [^{14}C]pinacolone and [^{14}C]Br_2Pin, showed similar activity to the same concentration (1 mM) of purchased BrPin in inactivating dilute (18 nM) Torpedo AcChE, 60% in 85 min; the purchased BrPin did not contain pinacolone or Br_2Pin.

Inhibition of Torpedo AcChE by TAP is competitive, $K_i = 0.075 \pm 0.001$ mM, as compared with 0.022 mM for commercial Electrophorus enzyme (17). DFP showed similar effectiveness in inactivating this affinity-purified Torpedo enzyme and Electrophorus AcChE, 2 μM leading to $t_{\frac{1}{2}}$ values of 12 and 14 min, respectively. Incubation of aliquots of enzyme with a deficiency of [^{3}H]DFP for 160 min, (a) alone and (b) in the presence of 20 mM TAP, led (a) to 56% and (b) to about 6% inactivation, and, after dialysis, bound counts were 10,300 and about 500 cpm, respectively. TAP protects effectively against DFP. Aliquots of enzyme were treated with 0.7 mM [^{14}C]BrPin for 10 min (a) alone, leading to 30% inactivation; (b) in the presence of 22 mM TAP, leading to no inactivation, and (c) after prior complete inactivation by DFP. Aliquots subjected to SDS-PAGE and auto-radiography showed major radioactivity at 70 kDa, diminished in the presence of TAP, but apparently unaffected by prior inactivation by DFP. The 70 kDa gel bands were counted, 810 dpm in (a), 450 dpm in (b), 45% decrease due to TAP, and 860 dpm in (c), no reduction by prior inactivation by DFP.

Effects of TAP in decreasing inactivation by [^{14}C]BrPin and introduction of ^{14}C were examined in more detail. Solutions (2.4 μM) containing 4.1×10^{-10} mol of active enzyme and (a) 1.0 mM [^{14}C]BrPin, (b) 1.0 mM [^{14}C]-BrPin and 26 mM TAP, and (c) no additives were allowed to stand for 120 min, leading to no loss in activity in (b) and (c), and in (a) to a 46% decrease, 1.9×10^{-10} mol inactivated. Solutions (a) and (b) were treated

with azide and subjected to SDS-PAGE and the 70 kDa bands were counted: 12,440 dpm, 3.4 x 10^{-10} mol [^{14}C]BrPin in the absence of TAP, 1.8 ^{14}C incorporated per enzyme unit inactivated, and 4700 dpm in the presence of TAP, a decrease of 2.1 x 10^{-10} mol ^{14}C, 1.1 ^{14}C per enzyme unit protected by TAP from inactivation.

In a similar experiment, solutions (1.26 μM) containing 2.15 x 10^{-10} mol of active enzyme, 1.0 mM [^{14}C]BrPin and 0, 15, 25 and 40 mM TAP were allowed to stand for 90 min, leading in the absence of TAP to 56% inactivation, 1.20 x 10^{-10} mol inactivated. In the presence of the TAP about 5% inactivation occurred, 0.1 x 10^{-10} mol of enzyme. After SDS-PAGE the 70 kDa bands were counted: 8300 dpm in the absence of TAP, 2.3 x 10^{-10} mol ^{14}C, 1.9 ^{14}C per enzyme unit inactivated; 4400-4800 dpm in the presence of 15, 25, and 40 mM TAP, a decrease of 3900-3500 dpm, 1.05-0.95 x 10^{-10} mol ^{14}C, 0.9 ^{14}C per enzyme unit protected by TAP. Inactivation of 0.5 μM AcChE by 0.5 mM [^{14}C]BrPin was prevented similarly by 3.5 mM decamethonium, with 50% reduction of incorporation of ^{14}C.

The failure, above, of prior inactivation by DFP to prevent introduction of [^{14}C]BrPin was examined further, by inactivation with [^{3}H]DFP. Two 2.27 mL aliquots of 0.17 μM enzyme in pH 7.0 buffer were inactivated completely by 1.7 μM [^{3}H]DFP. One of these and a third aliquot, without [^{3}H]DFP, were made 0.4 mM in [^{14}C]BrPin for 3 hours, leading to 43% inactivation in the third. The three samples were dialyzed and aliquots were counted. That treated only with [^{14}C]BrPin showed 440 cpm, that treated with [^{3}H]DFP alone, 4100 cpm, and that treated with both, 5000 cpm. [^{14}C]BrPin had entered the DFP-treated enzyme without displacing the DFP moiety. The Ellman assays indicated 3.9 x 10^{-10} mol of enzyme, of which 1.7 x 10^{-10} mol was inactivated in the aliquot treated only with [^{14}C]BrPin, 8% of which, 1.3 x 10^{-11} mol, was counted. The ^{14}C count indicated 2.3 x 10^{-11} mol ^{14}C, 1.8 ^{14}C per enzyme unit inactivated.

In the reverse order of treatment, two aliquots of 0.6 μM Torpedo AcChE were taken; one was inactivated by incubation with 1.5 mM BrPin for 20 hr and both were then allowed to stand for 36 hr with 50 μM 2:1 DFP:[^{3}H]DFP. The solutions were dialyzed and aliquots were counted, showing 16,500 and 204,000 cpm, after preinactivation by BrPin and in the absence of BrPin treatment, respectively. A control dialysis of a solution of the [^{3}H]DFP containing no protein indicated that about 8% of the count was retained in the solution. Thus, the purified Torpedo AcChE showed essentially no incorporation of [^{3}H]DFP after inactivation by BrPin.

N-methylpyridinium ion (1.6 mM, 10 x K_i) showed protection of AcChE from Electrophorus against inactivation by BrPin (1 mM, 5 x K_i): 40% and 63% inactivation after 4 and 6 hr incubation in the absence of NMPy, 11% and 28% in its presence.

4. Purchased Electrophorus AcChE was examined briefly. SDS-PAGE showed broad protein stain, and autoradiography showed labeling by [^{3}H]DFP and [^{14}C]BrPin at about 50 kDa. Fluorometric analysis indicated 16% content of active enzyme. The slow phase after the burst corresponded to 0.85 nM M7HQ in 232 s at 8.0 nM initial enzyme, giving a decarbamylation rate constant of 4.6 x 10^{-4} s^{-1}, lit. 4.2 x 10^{-4} s^{-1} (33). Carbamylation with 0.475 μM M7CQ was first order, $k_{(app)}$ = 5.0 x 10^{-3} s^{-1}, k_{+2}/K_i = 1.1 x 10^{4} $M^{-1}s^{-1}$, and, if K_i = 10 μM (33), k_{+2} = 0.10 s^{-1}, lit. 0.084 s^{-1} (33). Hydrolysis by 0.16 nM enzyme of 0.0077-0.25 mM AcSCh, at pH 7.8, led to k_{cat} = 1.5 x 10^{4} s^{-1} and K_m = 0.088 mM, lit. k_{cat} = 1.5 x 10^{4} s^{-1}, K_m = 0.056 mM at pH 7.5 (43). The kinetic constants are similar to reported values, and, based as they are on the fluorometric assay, give confidence in our assay of the Torpedo enzyme.

B. Isolation and Sequencing of Labeled Peptides; Labeling of Cys-231

In another isolation of AcChE from electric organ of T. nobiliana by limited tryptic digestion and affinity chromatography, assays for protein and activity indicated 90% content of enzyme based on Mr 70,000.

When characterized by N-terminal sequence analysis, the first 10 residues were Asp-Asp-His-Ser-Gly-Leu-Leu-Val-Asn-Thr, identical with the NH_2-terminal sequence of the enzyme from T. californica (44). Thus, the amino terminus of AcChE was preserved during proteolytic release of the enzyme.

Inactivation by nonradioactive BrPin: Aliquots of AcChE (7.3 μM, 1.6 mL) were treated (i) alone, (ii) with 1.0 mM BrPin, and (iii) with 1 mM BrPin and 14 mM TAP. After 5 hr, treatment with BrPin resulted in 53% loss of activity, while less than 5% inactivation was observed for the untreated control or the sample incubated with BrPin and TAP. For each sample, inactivation was terminated with sodium azide, and the enzyme was denatured, reduced and carboxymethylated and then digested with trypsin as described above. When samples were fractionated by reversed-phase HPLC, the OD profiles of the control and BrPin-treated samples were essentially the same with notable exceptions being fractions 64 (Figs. 1A and 1B; *, 26% CH_3CN) and 75-77 (32% CH_3CN). A sharp peak at Fraction 64 of control was strongly decreased in the sample inactivated by BrPin, and, after inactivation, additional material appeared to elute in Fraction 76. In the OD profile of Fig. 1A, a small, sharp peak in Fraction 75 was well-resolved from the major peak in Fraction 77, while after inactivation with BrPin (Fig. 1B), the two peaks appeared to coalesce.

For each digest, material in Fractions 64 and 76 were characterized by N-terminal sequence analysis. For the untreated enzyme, material in Fraction 64 contained a clear primary

sequence beginning at Ala-222 and extending to Arg-242 (initial yield 141 ± 8 pmol, repetitive yield 87 ± 1%). Several other amino acids were present in the early cycles at ≈10-20% the level of the primary sequence, but they could not be readily assigned to specific peptides. For enzyme inactivated by BrPin, Fraction 64 also contained the peptide beginning at Ala-222 as primary sequence, but at reduced levels (initial yield 37 ± 3 pmol, repetitive yield 89 ± 1%). The unassigned PTH-amino acids were present at the same level as in the absence of BrPin. For each Fraction 64, Cys-231 was identified at the tenth amino acid as the PTH-derivative of S-carboxymethylcysteine (retention time, 8.5 min). The Ala-222 peptide was not detected in Fraction 76 of the untreated enzyme, which contained a mixture of four peptides, starting at Lys-52, Val-150, Met-175 and Asn-280, each detected at ≈20 pmol. For Fraction 76 of the sample treated with BrPin, each of those four peptides was present at the same level, and in addition the Ala-222 peptide appeared to be the primary sequence present. With exception of PTH-carboxymethyl-Cys in cycle 10, each of the expected amino acids was detected as the primary released amino acid in the 13 cycles sequenced. Quantification was difficult because many of the amino acids were common to one or more of the other four sequences. Based upon the levels of release of PTH-Ala (cycle 1, 50 pmol), Ile (cycle 2, 80 pmol), Gly (cycle 6, 24 pmol), Pro (cycle 8, 25 pmol), and Pro (cycle 10, 10 pmol) and the absence of those amino acids in the corresponding cycles of the control sample, the Ala-222 peptide was sequenced with an initial yield of 80 ± 18 pmol and a repetitive yield of 84 ± 6%. Although the PTH-derivative of carboxymethylcysteine was not detected in cycle 10, there was clear release of a substance with retention time of 29.1 min, i.e., a hydrophobic material eluting after PTH-Leu (retention time 27.0 min). In the sequence analyses of Fraction 76, its release was detected only at cycle 10 for samples of AcChE inactivated with BrPin. It was not detected when sequencing the corresponding fraction of AcChE not treated with BrPin, nor was it detected in analysis of any of the samples from Fraction 64 of the tryptic digest. The identity of this peak will be discussed later.

Inactivation by [^{14}C]BrPin

1. To further characterize the site of alkylation by BrPin, [^{14}C]BrPin was used to inactivate AcChE. Treatment of AcChE (2.3 μM, 17 nmol) with 0.6 mM [^{14}C]BrPin resulted in 30% inactivation after 8 hours, while for a parallel sample treated in the presence of 15 mM TAP, there was less than 5% loss of activity. After denaturation, reduction and alkylation, enzyme treated with [^{14}C]BrPin alone incorporated 48,960 dpm, with a reduction by 65% in the presence of TAP (16,830 dpm incorporated). When the trypsin digests were fractionated by reversed-phase HPLC, the absorbance profiles were very similar to those of Figure 1 (data not shown). For the [^{14}C]BrPin-inactivated sample, approximately

30% of the loaded ^{14}C was recovered in Fractions 75-76, with the remaining counts distributed at low level throughout the gradient (Fig. 2 (●)). For enzyme inactivated in the presence of TAP, the ^{14}C in Fraction 75-76 was reduced by 90% (Fig. 2 (○)). Fractions 75 and 76, containing specifically labeled material were combined and sequenced for 20 cycles. Like the corresponding fractions from material exposed to nonradioactive BrPin, the fraction contained multiple sequences. PTH-amino acids of the Ala-222 peptide were identified at each cycle with the exception of carboxymethyl-Cys in the tenth cycle that would correspond to Cys-231. In addition, peptides beginning at Lys-52, Glu-89, Val-150, Met-175, and Asn-280 were present. For the [^{14}C]BrPin sample, release of ^{14}C showed a clear peak at cycle 10 (Fig. 3 (●)) corresponding to Cys-231, release that was reduced by 80% for the sample inactivated in the presence of TAP (Fig. 3, (○)).

The presence of the Ala-222 peptide in Fractions 75-76 only for enzyme inactivated with BrPin, as well as the specific release of ^{14}C in cycle 10, indicates that BrPin inactivates AcChE by reacting with Cys-231 and that the reaction does not occur when TAP occupies the enzyme active site. However, quantitative analysis of the amounts of the Ala-222 peptide was not possible because of the presence of many other peptides in that fraction. Rather than carrying out further purification procedures to isolate [^{14}C]BrPin-labeled peptide from the other peptides, we used a radiochemical sequence strategy to prove that the ^{14}C released in cycle 10 was associated with Cys-231. We took advantage of the fact that there is a proline (Pro-229) at the amino terminal side of Cys-231 in the predicted peptide. o-Phthalaldehyde (OPA) reacts with primary amines but not with secondary amines, and it can be used at any cycle of Edman degradation to block the amino termini of all peptides except those with amino terminal proline (40).

2. AcChE (5 μM active enzyme; 20 nmol) was inactivated with [^{14}C]BrPin (1.2 mM) in the presence or absence of 15 mM TAP. In the absence of TAP there was 29% inactivation in 6 hr and 73% after 22 hr, while in the presence of TAP there was <5% inactivation after 6 hr and 38% after 22 hr. Thus, after 22 hours, TAP provided ≈50% protection against inactivation. Based upon counting of aliquots of [^{14}C]BrPin-inactivated enzyme after denaturation, reduction and alkylation, the presence of TAP reduced ^{14}C incorporation by 25%. As in the experiment of Figure 1, the HPLC absorbance profiles of tryptic digests of enzyme inactivated by [^{14}C]BrPin in the absence or presence of TAP were quite similar, except for a clear decrease of a sharp peak in Fraction 64 for the former sample (data not shown). The distribution of ^{14}C in the gradient fractions was similar to that of Figure 2, with 25% of recovered counts in Fraction 76 (Fig. 4). For the sample inactivated in the presence of TAP, ^{14}C in Fraction 76 was reduced by 50%. When material in Fraction 64 was sequenced, the Ala-222 peptide was the clear primary sequence in

each sample and it was present in twofold higher abundance in the sample protected with TAP (initial yield 125 ± 5 pmol, repetitive yield 88 ± 1%) than in the unprotected sample (initial yield 58 ± 2 pmol, repetitive yield 86 ± 1%). In contrast, for Fraction 76 the Ala-222 peptide was present at twice the level in the unprotected sample (127 ± 10 pmol) than in the protected sample (59 ± 1 pmol), Table I.

Material in Fraction 76 of the unprotected sample was divided into equal aliquots for sequence analysis with and without the use of OPA. When an aliquot was sequenced for 22 cycles without exposure to OPA, there was release of ^{14}C in cycle 10, as well as a low level of release in cycles 6 and 7 (Fig. 5(▽)). For a sample reacted with OPA after cycle 7 (Fig. 5(●)) release of ^{14}C in cycle 10 remained, while for a sample reacted with OPA after the second cycle, release at cycle 10 was prevented (Fig. 5(x)). In the last case the release of ^{14}C observed at cycle 3, which was not seen in either of the other samples, presumably resulted from the additional exposure to trimethylamine associated with the reaction with OPA. As expected, the samples contained amino acids consistent with the presence of five peptides, with the Ala-222 peptide as the principal peptide. Treatment with OPA after the second cycle prevented subsequent sequencing of any of the peptides, while for the sample reacting with OPA after cycle 7, only the peptide beginning at Ala-222 continued to be sequenced (Table I), and released amino acids could be quantified through Gly-241. Thus the results of the radiosequence analysis confirm that the release of ^{14}C in cycle 10 must be due to alkylation of Cys-231 by [^{14}C]BrPin.

As noted during the sequence analysis of Fraction 76 of the tryptic digest of AcChE inactivated by nonradioactive BrPin, release of PTH-carboxymethylcysteine was not seen at cycle 10. However, for the sample not treated with OPA and for the sample treated with OPA after cycle 7, there was clear release of a substance with a retention time of 29.1 min, i.e., a hydrophobic substance eluting after PTH-Leu. For the sample treated with OPA, it was the only release detected in cycle 10, a result indicating that this should be a product of the reaction of BrPin with cysteine.

Reaction of BrPin with L-Cysteine. Two approaches were used to characterize the reaction of BrPin with cysteine. First, the reaction of L-Cys with BrPin in methanol was compared with that of glycine. Glycine HCl failed to react with BrPin, indicating lack of reactivity of the α-amino group, while L-Cysteine HBr yielded a reaction product with the elemental analysis expected for S-pinacolonyl cysteine hydrobromide. As noted in Experimental Methods, the compound was not stable in solution, and NMR confirmation of the product was not completely successful. When 2 nmol of product were subjected to N-terminal

sequence analysis, two peaks were detected, a major peak eluting at PTH-Tyr (retention time, 16.7 min) and a minor peak eluting at 29.0 min, i.e., the same retention time as seen in the sequence analysis of the Ala-222 peptide from enzyme inactivated with BrPin. Secondly, cysteine was reacted with BrPin under conditions similar to an inactivation and the reaction product was isolated by HPLC (see Experimental Methods). When the product was subjected to N-terminal sequence analysis, once again products were detected at 16.7 and 29.0 min.

Treatment with Sulfhydryl Reagents: Since BrPin is shown by the sequencing studies to inactivate AcChE from T. nobiliana by alkylating Cys-231, thiol-specific reagents were then examined as inactivators. 2,2'-Dipyridyldisulfide (2-PDS) proved to be the most effective (Table II). At 33 μM it inactivated 0.80 μM AcChE by 66% after 18 hr incubation, and the presence of the competitive inhibitor TAP (14 mM) completely prevented this inactivation. At lower concentrations the purified enzyme is less stable and more rapidly inactivated. At 0.04 μM enzyme, 22 μM 2-PDS led to 93% inactivation in 1.7 hr; 22 μM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), also containing aromatic binding groups, but negatively charged, was much less effective, leading to 18% inactivation in 1.7 hr; enzyme alone at this lower concentration (0.04 μM) appeared to lose ~6% activity. Iodoacetamide (IAm) was still less effective: at 230 μM, it led to 60% inactivation of 0.017 μM enzyme in 22 hr, rather little more than the spontaneous loss of 40% enzyme activity under these conditions. In this case presence of TAP appeared to reduce loss of activity below the spontaneous rate. Iodoacetate (IAc), negatively charged, showed no inactivation beyond the spontaneous under the same conditions. While 22 μM 2-PDS effectively inactivated AcChE from T. nobiliana, unlike BrPin it did not inactivate enzyme from Electrophorus; IAm and IAc (230 μM) also caused no loss of activity of this enzyme.

Effects of 2-PDS on AcChE from T. nobiliana were examined in further detail. It acts initially as an effective reversible competitive inhibitor, $K_{i(com)}$ = 39 ± 3 μM (Fig. 6A). In contrast, it is a much less effective and largely noncompetitive inhibitor of AcChE from Electrophorus, $K_{i(com)}$ = 3.2 ± 0.4 mM, $K_{i(nonc)}$ = 1.2 ± 0.2 mM (Fig. 6B). Results of study of remaining activity after periods inactivation of enzyme from T. nobiliana by a range of concentrations of 2-PDS corrected for spontaneous deactivation, are summarized (Fig. 7). Slopes of log % activity vs. time lead to values of rate constants for inactivation, k (Fig. 8). The observed hyperbolic dependence of k upon 2-PDS concentration is consistent with a mechanism of reversible binding ($K_i = k_{-1}/k_{+1}$) followed by inactivation k_2 (eq. 4), which predicts an observed first-order rate constant for inactivation, k (eq. 5).

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot I \xrightarrow{k_2} EI \qquad (4)$$

$$k = \frac{k_2 [2\text{-PDS}]}{K_i + [2\text{-PDS}]} \qquad (5)$$

Computer fitting of k vs. [2-PDS] leads to $K_i = 28 \pm 3$ μM, close to that found by reversible inhibition, and $k_2 = 5.0 \pm 0.2 \times 10^{-4}$ s^{-1}. A similar treatment of inactivation by a range of concentrations of BrPin leads to observed first-order rate constants, k, well fit by a hyperbolic function (Fig. 9), with $K_i = 0.18 \pm 0.02$ mM, $k_2 = 1.8 \pm 0.1 \times 10^{-4}$ s^{-1}.

Protection by TAP against inactivation of AcChE from T. nobiliana by 2-PDS was examined and results are summarized in Fig. 10. At 56 μM 2-PDS, AcChE was inactivated with a rate constant of 3.0×10^{-4} s^{-1}, 72% in 70 min. Addition of 0.5, 1.0 and 3.0 mM TAP, concentrations equal to 2.5, 10, and 15 x K_i, decreased inactivation to 45, 20, and 10% in this period, with rate constants 1.5×10^{-4}, 0.5×10^{-4} and 0.27×10^{-4} s^{-1} respectively.

Since inactivation by BrPin and by 2-PDS were both inhibited by TAP, and 2-PDS is a thiol-specific reagent while BrPin is a more general alkylating agent, the effect of prior inactivation by 2-PDS on subsequent introduction of [^{14}C]BrPin was examined. Solutions of (i) enzyme only and (ii) enzyme made 67 μM in 2-PDS were kept at 4°C for 4 days, (i) remaining stable, (ii) losing essentially all of its activity. They were then made 0.6 mM in [^{14}C]BrPin, and kept at 25°C for 8.5 hr. Enzyme (i) lost 30% of its activity and, on being worked up showed the effects of reaction with [^{14}C]BrPin on its HPLC profile as before. In comparison, preinactivation by 2-PDS led to exclusion of 80% of the ^{14}C. After trypsin digestion and HPLC there were low ^{14}C counts distributed essentially uniformly over the gradient with no increase in the fraction 75-77 region.

The effect of inactivation by BrPin or 2-PDS in preventing subsequent reaction with the active-site carbamylating agent, M7CQ, was examined briefly. Treatment with M7CQ of aliquots of AcChE (i) inactivated 65% by BrPin, and (ii) 34% by 2-PDS, indicated 35% residual activity in (i) and 62% in (ii).

Effects of BrPin and sulfhydryl reagents on AcChE from other sources were examined briefly. BrPin, 0.8 mM, also inactivated enzyme from human erythrocytes, as it did that from Electrophorus, while 2-PDS did not. Neither BrPin, 1 mM, nor 2-PDS, 0.2 mM, inactivated horse serum or human serum butyrylcholinesterase (BuChE) in incubations followed over 4.5 hr

at 25°C and 3 days at 4°C for both compounds. BrPin acts as a reversible inhibitor for human BuChE, $K_{i(com)}$ 0.5 mM, $K_{i(nonc)}$ 1.5 mM.

C. Inactivation of AcChE by Phenacyl Bromide

1. Synthesis of PhABr from acetophenone in a 100-μmol scale has been worked out, and its synthesis as a radioactive labeling agent is feasible. It acts initially as a reversible inhibitor (Fig. 11), ($K_{i(com}$ = 1.7, $K_{i(nonc)}$ = 1.4 mM) and then inactivates.

2. Protection against inactivation by active-site-directed reversible inhibitors has been examined (Table III). PTA offers complete protection to 50% inactivation, less protection on longer incubation. N-Methylpyridinium ion (NMPy), 5-N,N-diethyl-N-n-butylammonio-2-pentanone (DEBAP), and TAP offer partial protection initially. 3-Dimethylaminopyridine (3-DMAPy) offers partial protection initially, and little on long incubation. TEMA offers essentially no protection. The above results are with enzyme from Electrophorus.

AcChE from T. nobiliana was inactivated much more rapidly by PhABr; appropriate conditions for inactivation and protection, and thus of labeling this enzyme were observed (Table III).

The effect of prior inactivation by PhABr on subsequent introduction of ^{14}C from [^{14}C]BrPin was examined. AcChE from T. nobiliana, 1.5 μM, was inactivated completely by incubation for 21 hr with 0.8 mM PhABr. A control was stable. Aliquots of each were incubated with [^{14}C]BrPin, 1.0 mM, for 6 hr leading to 20% inactivation in the control, 0.15 nmol inactivated; 1.9 ^{14}C was introduced per unit of active enzyme inactivated, and prior inactivation by PhABr excluded 71% of the ^{14}C. Enzyme inactivated spontaneously by long standing at room temperature excluded a similar proportion of ^{14}C.

3. AcChE from other sources:

a. AcChE from human erythrocyte (Sigma) was examined at pH 7.0, phosphate buffer, I = 0.2. The enzyme was centrifuged and the supernatant was incubated (i) alone (ii) with 0.37 mM PhABr and (iii) with 0.37 mM PhABr and 20 mM TAP. Inactivation was slow, 40% after 18 hr, in (ii), 12% in (iii), 70% protection by TAP; enzyme alone was stable.

b. Action of 0.5 mM PhABr on human butyrylcholinesterase (Sigma Chemical Co., St. Louis, MO) was examined, pH 7.0, phosphate buffer, I = 0.2. Aliquots, from 1 unit in 0.5 mL were incubated (i) alone, (ii) with 0.5 mM PhABr, (iii) with 0.5 mM PhABr plus 7 mM TAP: (i) was stable; (ii) lost 32% and 72% after 3.25 and 19 hr, respectively; (iii) was protected.

4. An alternative course toward radioactive labeling with α-haloketones, inactivation by nonradioactive reagents followed by reduction of the carbonyl with $[^3H]BH_4^-$, was considered.

AcChE from T. nobiliana was inactivated 68% by BrPin, 18% by BrPin in the presence of 15 mM TAP, and then the samples and a control were treated with $[^3H]BH_4^-$, 490 Ci/mole, at pH 8 for 30 min. The inactivated enzyme had only 14% more ^3H than the control, the TAP protected enzyme about 7% more than the control. $[^3H]BH_4^-$ did not reduce the pinacolonyl carbon significantly in the inactivated intact enzyme. The experiment was repeated with denaturation of the enzyme prior to treatment with $[^3H]BH_4^-$. Enzyme was inactivated 77% by BrPin, 6% in the presence of 12.5 mM TAP. Treatment with $[^3H]BH_4^-$ after denaturation introduced 1.24×10^5 dpm/nmol in a control, 2.36×10^5 dpm/nmol in the 77% inactivated sample, 1.45×10^5 dpm presumably due to reduction of 0.77 nmol of introduced carbonyl.

The isotope effect was then examined in reduction of acetophenone. A solution (1 mL) of 0.18 mM acetophenone was reduced completely to α-phenylethyl alcohol by 1.0 mM $[^3H]BH_4^-$ in 1.5 hr, as indicated by HPLC. The HPLC fractions containing ^3H, ~ 0.18 nmol had 2.8×10^5 dpm, 1.3 μCi, as compared with 8.8 μCi calculated in 0.18 nmol of 490 Ci/mole $[^3H]BH_4^-$, indicating an isotope effect of 6.8 in the reduction. This leads to a calculated value of 9.8×10^3 dpm or 0.91 nmol reaction with $[^3H]BH_4^-$ in 0.74 nmol of enzyme inactivated by BrPin, 1.2 pinacolonyl group introduced per enzyme unit inactivated. About two such groups have been observed in inactivation by $[^{14}C]$BrPin.

AcChE was then labeled with $[^{14}C]$BrPin in the absence and presence of TAP, leading to 15,000 and 11,000 dpm in the two samples, respectively. Treatment with 1.0 mM $NaBH_4$ for 10 min reduced the counts to 7,300 and 5,500 dpm, respectively, indicating that about half the pinacolonyl label is sufficiently labile to be displaced by the strongly nucleophilic hydride.

Aliquots of enzyme from T. nobiliana, 0.5 μM, 0.28 nmol (i) completely inactivated in 1.5 hr by 0.5 mM PhABr, (ii) 90% protected by 19 mM TAP and (iii) untreated, were denatured and treated with 0.15 mM $[^3H]BH_4^-$, and counted; the results were (i) 1.46×10^5 dpm, (ii) 1.05×10^5 dpm, and (iii) 1.18×10^5 dpm, indicating about 2-3 times as much ^3H in the enzyme as in the reduced carbonyl.

Another treatment was carried through to HPLC of the peptides. Aliquots of 3.2 μM enzyme, 2.1 nmol were (i) inactivated 77% by 0.5 mM PhABr in 2 hr; (ii) inactivated 17% in the presence of 11 mM TAP; and (iii) untreated and stable, were denatured, reduced and alkylated, and treated with 1 mM $[^3H]BH_4^-$ for 1.5 hr, and counted: (i) 4.02×10^6 dpm, (ii) 3.88×10^6 dpm and (iii) 3.46×10^6 dpm. After tryptic digestion HPLC indicated

that significant ^{3}H reduced TAP may not have been dialyzed away. Counts in HPLC fractions 11-19, (i) 5.5×10^5 dpm (ii) 5.5×10^5 dpm and (iii) 2×10^5 dpm, may reflect reduction of nonspecific phenacyl in (i) and (ii), not protected by TAP, and reduction of enzyme in (iii); counts in HPLC fractions 20-21 were (i) 3.2×10^5, (ii) 2.0×10^5 and (iii) 0.7×10^5, indicating specific labeling in (i), decreased by TAP in (ii), 1.2 phenacyl introduced in fractions 20-21 per enzyme unit inactivated, and 0.6 phenacyl excluded per enzyme unit protected.

D. Inactivation of AcChE by Methyl Benzenesulfonate (MBS)

MBS, an aromatic alkylating agent delivering a small methyl group to a nucleophile, is also under consideration as a possible labeling agent. Its synthesis from benzenesulfonyl chloride and methanol has been worked out on a 100-μmol scale, and use of radioactive methanol to produce a labeling agent is feasible.

It acts initially as a moderately effective reversible inhibitor for AcChE from Electrophorus, $K_{i(com)}$ 5.9 mM, $K_{i(nonc)}$ 21 mM, Fig. 12, and then it inactivates (Table IV). This inactivation is strongly retarded by N-methylacridinium ion, 3-acetylpyridine, and 2-dimethylaminopyridine, during the initial period, i.e., to 50-75% inactivation. N-Methylpyridinium ion is less effective; propidium is still less effective, while, remarkably, TAP, PTA and trimethylammonioacetophenone offer no protection.

Effect of prior inactivation by MBS on subsequent introduction of ^{14}C from [^{14}C]BrPin was examined. Inactivation of 0.6 nmol of AcChE from T. nobiliana by MBS was carried out at 4°C for 4 days, a control remaining stable. On treatment with 1 mM [^{14}C]BrPin for 9 hr the active enzyme lost 17% activity, 0.1 nmol of enzyme inactivated, and showed 5000 dpm. The MBS inactivated enzyme, treated in the same way with [^{14}C]BrPin, showed 1500 dpm.

E. Binding to AcChE-Substrate Complex in Noncompetitive Inhibition and in Inhibition by Substrate

Six substrates, β-substituted ethyl acetates, $X\text{-}CH_2CH_2OCOCH_3$ of varied reactivity were studied; three cationic, AcCh, N,N-diethyl-N-n-butyl-ammonioethyl acetate (DEBAAc) and N-methylammonioethyl acetate (MAAc); two nonpolar, DMBAc and n-butyl acetate (n-BAc), and one polar uncharged, methylsulfonylethyl acetate (MSAc). Data for enzymic reactivity of the substrates and the steady-state ratio of EA to E·S are summarized in Table V. Values of k_{cat} were based on $k_{cat} = 1.6 \times 10^{-4}$ s^{-1} for AcCh.

Studies were carried out at pH 7.8, and one substrate, MAAc, was studied at pH 6.5 because of possible nonenzymic hydrolysis at higher pH; AcCh was also studied at this pH for comparison,

showing decreased k_{cat}, and no change in K_m. DMBAc has favorable k_{cat}, but less favorable binding than its cationic isostere, AcCh. Isosteric substrates with small β-substituents, uncharged n-BAc and cationic MAAc, have lower k_{cat} and less favorable K_m values. The cationic substrate with a large β-substituent, DEBAAc, has low k_{cat} despite favorable, poorly productive binding. MSAc, containing a polar β-substituent with no net charge, isosteric with AcCh and DMBAc, has both low k_{cat} and poor binding.

Two sets of values of k_2 were calculated (Table V), based on the k_{cat} values and the equality: $k_{cat} = k_2k_3/(k_2 + k_3)$. One is based on $k_2 = 6 \times k_3$ for AcCh (45) derived from study of the 11-S lytic form, leading to $k_3 = 1.9 \times 10^4\ s^{-1}$, the other based on $k_2 = 2.1 \times k_3$ for AcCh (46) derived from study of the 18-14 S form, leading to $k_3 = 2.4 \times 10^4\ s^{-1}$. The k_2 value for AcCh based on $k_2 = 6 \times k_3$ is significantly larger than that based on the smaller factor, while the slower substrates, in which k_2 becomes rate limiting, show small, if any, differences in the two sets. The same value of k_3 applies to all acetate substrates. The relative amounts of EA and E·S present during hydrolysis may be calculated from the steady-state condition (eq. 6):

$$d[EA]/dt = 0;\ k_2[E\cdot S] = k_3[EA],\ [EA]/[E\cdot S] = k_2/k_3 \quad (6)$$

For AcCh alone, [EA] exceeds [E·S], and is present at 85% or 68% of total enzyme at saturation at pH 7.8, depending on the value of k_2 used. For DMBAc, [E·S] is present as 67% or 57% of total enzyme at saturation. For all the other substrates, [E·S] far exceeds [EA], and comprises about 90% of total enzyme at saturation.

The inhibitors studied and values of binding constants are given in Table VI. Not all possible combinations with the varied substrates were examined. tert-Butylammonium ion, IA, an effective competitive inhibitor, $K_{i(com)} = 0.7$ mM, showed stronger noncompetitive inhibition of the slower substrates, DMBAc, DEBAAc and MSAc, $K_{i(nonc)} = 1.6$ mM, than of the more reactive AcCh, $K_{i(nonc)} = 7$ mM. Its lower homologue, isopropylammonium ion, IB, a less effective competitive inhibitor, $K_{i(com)} = 1.9$ mM, showed similar unfavorable $K_{i(nonc)}$ values for AcCh and DEBAAc, 23 and 19 mM, and more favorable values for DMBAc, 1.2 mM, and n-BAc and MSAc, 6 mM.

In the second homologous pair, TeMA and TrMA, IIA and IIB, the tetrasubstituted TeMA also showed more favorable $K_{i(nonc)}$ values in hydrolysis of the slower substrates, DMBAc, n-BAc, MAAc and MSAc, 3-5 mM, but not in hydrolysis of DEBAAc, 11 mM, as compared with 7.5 mM for AcCh. The trisubstituted TrMA showed a contrary effect, stronger $K_{i(nonc)}$ in hydrolysis of AcCh than with the slower substrates, MAAc, DEBAAc and MSAc, but not in hydrolysis of DMBAc.

Pairs of quaternary and tertiary ammonio compounds more closely related in structure to AcCh were then examined. Choline, IIIA, showed more favorable $K_{i(nonc)}$ values in hydrolysis of the slower substrates, DMBAc, n-BAc, DEBAAc and MSAc, 2-4 mM, than with AcCh, 7 mM, but apparently not with MAAc at pH 6.5. Its lower homologue, IIIB, showed weaker $K_{i(nonc)}$ in hydrolysis of slower substrates, n-BAc, MAAc, DEBAAc and MSAc, 7 mM, than with AcCh, while values for AcCh and DMBAc were similar, ~2.7 mM. Competitive inhibition is stronger at pH 7.8 than at pH 6.5, and for IIIA than for IIIB. Choline ethyl ether, IVA, showed more favorable $K_{i(nonc)}$ in hydrolysis of the less reactive substrates, DMBAc and n-BAc, 3.9 and 0.7 mM, than with AcCh, 9.6 mM; again, the lower homologue, IVB, showed the reverse effect, more favorable $K_{i(nonc)}$ for AcCh, 0.26 mM, than for DMBAc, n-BAc and DEBAAc, 0.65-2.1 mM; the tetrasubstituted compound was the more effective competitive inhibitor. The amide analogue of AcCh, acetamidocholine, VA, and its lower homologue, VB, both showed more effective noncompetitive inhibition of the slower substrates DMBAc and DEBAAc than of AcCh, 2.6 and 7.5 mM, as compared with 11 mM, and 1.7 and 3.2 mM, as compared with 4.7 mM, respectively.

Tetraethylammonium, VI, also showed more favorable $K_{i(nonc)}$ in hydrolysis of the slower substrate, DMBAc, 0.3 mM, than with AcCh, 2.1 mM, similar to that for DEBAAc. The larger tetra-n-propylammonium ion, VIIA, has very favorable $K_{i(nonc)}$ for both AcCh and DMBAc, 0.13 and 0.16 mM, similar to its competitive binding, and less favorable $K_{i(nonc)}$ with DEBAAc, 0.54 mM. The tertiary analogue, tri-n-propylammonium ion, VIIB, also has favorable $K_{i(nonc)}$ for AcCh and DMBAc, 0.3 and 0.5 mM, and substantially less favorable $K_{i(nonc)}$ with DEBAAc, 7.9 mM.

The ketone analogue of AcCh, 5-trimethylammonio-2-pentanone, VIIIA, the most effective competitive inhibitor in this series, $K_{i(com)}$ = 0.035 mM, showed no noncompetitive inhibition in hydrolysis of AcCh, while its tertiary homologue, VIIIB, did, 2.5 mM for AcCh, 3.6 mM for DMBAc. The quaternary ketone, IXA, with larger β-triethylammonio substituent, had less favorable $K_{i(com)}$, 0.5 mM, than the lower trimethyl homologue, VIIIA, and a noncompetitve component $K_{i(nonc)}$, 2.7 mM; its tertiary diethyl analogue, IXB, showed similar $K_{i(com)}$ and less favorable $K_{i(nonc)}$, 7.4 mM. The ketone with the larger, diethyl-n-butylammonio β-substituent, X, had more favorable $K_{i(nonc)}$, 0.3 mM, with AcCh, 0.2 mM with DMBAc. The ester substrate, DEBAAc, XI, with the same large β-substituent, having low k_{cat}, could be examined as an inhibitor for AcCh. It is an effective noncompetitive inhibitor, $K_{i(nonc)}$ = 0.87 mM, and less effective competitively, $K_{i(com)}$ = 2.2 mM.

Compounds containing two terminal tert-butyl groups were prepared, analogues of decamethonium, for study as uncharged reversible inhibitors which might occupy the active and peripheral binding sites: triethylene glycol di-tert-butyl

ether, and the dipivaloate ester of N-methyldiethanolamine; the tert-butylacetate ester of N-methyldiethanolamine, in an attempt to make the diester, and the diacetate of N-methyldiethanolamine for comparison. This was not pursued further when Dr. Howard left the project for a teaching position.

F. Pyridine Derivatives as Reversible Inhibitors and Inactivating Agents

1. Results of titrimetric study of reversible inhibition by pyridine derivatives of hydrolysis of AcCh by AcChE from Electrophorus are summarized in Table VII. Pyridine itself has $K_{i(com)}$ 8.1 mM, and is largely competitive. Binding is increased by aliphatic substituents, t-butyl, vinyl and epoxide, by electron withdrawing substituents, acetyl, bromoacetyl and nitro, and by electron donating methoxy and amino. It is very strongly increased by dimethylamino groups to $K_{i(com)}$ = 0.05-0.1 mM, slightly stronger binding than positively charged N-methylpyridinium ion, $K_{i(com)}$ = 0.16 mM; N-methylation further strengthens binding of dimethylamino compounds. Binding is also increased by decreasing pH, increasing the proportion of protonated pyridine present. Binding is little, if at all, affected by conversion of pyridine or 4-nitropyridine to the N-oxides. Pyridine and the alkylpyridines have pKa values < 6 and are essentially unprotonated at pH 7.8; 2-aminopyridine, pKa 6.96, raised to 7.8 by a 4-CH_3 group, and 3, 2 and 4-dimethylamino pyridines, with pKa values of 7.0, 6.5 and 9.7, respectively, are significantly protonated, which contributes to their binding.

The alkylpyridines, the N-oxide, 2-methoxypyridine, and 3-aminopyridine at pH 7.8, 2-amino-4-methylpyridine and 2-dimethylaminopyridine at pH 7.8 and 9, and 3-dimethylaminopyridine at pH 7.8 and 6.5, act largely competitively; the acetylpyridines, the nitropyridines, 2-aminopyridine at pH 7,8, 3-aminopyridine and 3-dimethylaminopyridine at pH 9, 4-dimethylaminopyridine and the N-methylpyridinium ions all show comparable competitive and non-competitive binding.

2. Noncompetitive inhibition as a function of substrate reactivity was examined for some benzene and pyridine derivatives in hydrolysis of AcCh and DMBAc. 3-Acetylpyridine, N-methylpyridinium ion, acetophenone, nitrobenzene and 3-trimethylammoniophenol show the same noncompetitive values in inhibition of the two substrates (Table VIII). 2-Dimethylaminopyridine and 3-tert-butylphenol are stronger noncompetitive inhibitors of DMBAc than AcCh, with $K_{i(nonc)}$ values of 0.11 and 0.66 mM for DMAPy and 0.023 and 0.17 mM for the phenol, for DMBAc and AcCh, respectively.

3. A brief comparison of reversible inhibition by aryl compounds of AcChEs from T. nobiliana and Electrophorus was made, Table IX. 4-tert-Butylpyridine and PTA inhibit enzyme from T. nobiliana somewhat more strongly, $K_{i(com)}$ 0.30 mM vs. 1.3 mM, 0.025 mM vs. 0.086 mM, respectively, and both act largely competitively. 2-DMAPy binds similarly to the two enzymes, $K_{i(com)}$ 0.1 mM, and also is largely competitive. 3-AcPy is a weaker inhibitor of T. nobiliana than Electrophorus, 12 mM vs. 2.4 mM, both competitively and noncompetitively.

Several pyridine derivatives, potential inactivating agents, 3-chloromethylpyridine, 2- and 4-vinylpyridine, 2-pyridyloxirane, and 2-PDS, each 3.0 mM, were examined with 5 mM Electrophorus AcChE, 24 hr incubation, and caused no inactivation.

3-Bromoacetylpyridine (BAPy), 3 mM, 1.5 x $K_{i(com)}$, 1.7 x $K_{i(nonc)}$ inactivated AcChE from Electrophorus (Table X) with effectiveness similar to PhABr, Table III. NMPy, 3-AcPy, and PTA, at 10 x K_i offered moderate protection, 30-40%; DMAPy, TMAAPh, TAP, TeMA and DeMe offered less protection, 10-20%, and tubocurarine offered none.

VI. Discussion

A. Labeling of AcChE from T. Nobiliana by [^{14}C]BrPin

Our studies of the kinetics of hydrolysis of cationic and uncharged substrates led us to the conclusion that the subsite at which the cationic group of AcCh binds is not anionic, and is uncharged and hydrocarbon in character (11). Our studies of effects of cationic and uncharged aliphatic reversible inhibitors related in structure to AcCh led to the conclusion that these cationic and uncharged groups bind at the same subsite (13, 16). However, it has been argued that there are separate binding sites, anionic and lipophilic, for the isosteric trimethylammonioethyl and dimethylbutyl substituents (8). Thus, in study of AcChE covalently modified by reaction of the active-center serine with β-trimethylammonioethyl and 3,3-dimethylbutyl methylphosphonofluoridates, the uncharged conjugate was reactivated more rapidly by cationic oximes, and bound quaternary cations more strongly than the cationic conjugate. This was taken as support for separate binding sites for the cationic and neutral substituents in the inactivators. However, the two conjugates are quite different species. Covalent binding of a cationic charge to the active-site serine could cause conformational change and also repel and reduce the binding and reactivity of cationic reagents.

Evidence for binding of tert-butyl and trimethylammonio substituents at the same site is also found in binding of benzene derivatives (24). A hydrophobic site has been proposed, at which aryl groups bind (47). However, we found that substituted

benzenes and phenols bind via a polar interaction, with energies consistent with the Hammett equation; further, a single relation covered both cationic and uncharged polar substituents, with no additional effect that might be attributed to cation-anion interaction (24). This binding, probably involving charge-transfer interaction with aromatic amino acid side chains, is strongly increased by electron withdrawal, ρ = +2, and the substituents have their effects by electron withdrawal from or donation to the aromatic rings. This aryl site is contiguous with the trimethyl cavity, and uncharged tert-butyl and dimethylamino, and cationic trimethylammonio substituents on aryl nuclei bind there. Notably, in the binding of phenols, meta-tert-butyl and meta-trimethylammonio substituents make equal large synergistic contributions to binding, each 4 kcal/mol higher than the very different bindings of the related substituted benzenes, affected as they are by the very different σ-values of the two substituents. That these large effects require the same meta orientation of these cationic and uncharged substituents indicates that the substituents interact at the one trimethyl site.

Our conclusions about the character of the active site were recently proved accurate by the X-ray diffraction study of AcChE from T. californica which characterizes the binding site as a "gorge" lined with aromatic groups (26). Since there are multiple excess negative charges on the enzyme surface (15) and we had concluded that the binding site is uncharged, we deemed uncharged reagents to be more suitable for specific labeling of the active site. 1-Bromopinacolone, BrPin, and phenacyl bromide, PhABr, are two such compounds; initial emphasis has been placed on [^{14}C]BrPin, synthesized from freshly prepared $CH_3{}^{14}COCl$ and $(CH_3)_3CMgCl$, followed by bromination (27).

Our kinetic studies had been carried out on commercially available (Sigma) AcChE from Electrophorus. The AcChE from Electrophorus (Sigma) was impure, containing much (>80%) extraneous protein and the AcChE labeled by [^{3}H]DFP was itself not homogeneous, as shown by gel electrophoresis. For labeling studies purified enzyme was required. Electric organ from T. nobiliana was available to us, and although AcChE from this source had been little studied, we chose to use it (27). While there proves to be substantial homology in the primary structres of AcChE from these different sources, there is an interesting difference which will be discussed below.

AcChE, isolated from T. nobiliana, assayed by fluorescent burst of M7HQ, has K_m and k_{cat} values in hydrolysis of AcSCh similar to the respective values reported for enzymes from T. marmorata and T. californica, with slightly more favorable K_m and less favorable k_{cat}. The k_{cat} value is 0.3 that of Electrophorus enzyme; the decarbamylation rate constant is also 0.3 that for decarbamylation of Electrophorus enzyme, while the

carbamylation constants appear to be the same (27). If deacetylation in hydrolysis of AcSCh is also 0.3 as great in Torpedo as in Electrophorus enzyme, this would account for the difference in their hydrolytic reactivities.

Our earlier study of inactivation of AcChE from Electrophorus by unlabeled BrPin and protection against it by reversible inhibitors structurally related to AcCh (17) led logically to use of TAP in study of protection against [^{14}C]BrPin.

TAP is cationic and isosteric with AcCh and binds in the active site like the natural substrate. It is an effective competitive inhibitor, and also inhibits inactivation by DFP. Its effect in protecting against inactivation by [^{14}C]BrPin and excluding ^{14}C provided an informative comparison of the binding of the tert-butyl of BrPin and the trimethylammonio group of AcCh. That TAP decreases incorporation of 0.9-1.1 ^{14}C per enzyme unit which it protects supports the view that its trimethylammonio group, as well as that of AcCh, and the tert-butyl group of one BrPin, bind in the trimethyl cavity; BrPin alkylates a nucleophile from that position in the active site, and would be an informative labeling agent (27).

Other α-haloketones have been studied. Bromoacetone, lacking the trimethyl structure, did not inactivate AcChE (48). 1-Bromo-3-trimethylammonioacetone, with one more methylene than BrPin, showed mixed inhibition and inactivated AcChE (49). It does not appear to have been studied as a radioactive label. Surprisingly, 3-bromo-5-trimethylammonio-2-pentanone, the bromo derivative of TAP, closely related to AcCh and a very effective reversible inhibitor, did not inactivate AcChE (48). α-Halocarbonyl compounds with appropriate substituents inactivate serine proteases by alkylating imidazole of active-site histidine (50). The alkylation proceeds via addition of the serine hydroxyl to the carbonyl of the reagents (51), and does not occur with anhydrochymotrypsin, which lacks the hydroxyl (52). The reaction of BrPin with AcChE does not involve the serine hydroxyl, since incorporation of ^{14}C occurs with DFP-inactivated enzyme, in which the serine hydroxyl is phosphorylated, and reaction of [^{3}H]DFP-inactivated enzyme with BrPin does not displace the label (27).

An isopropyl group of DFP binds at the trimethyl site, since its reaction is inhibited by cations which bind there (5), and the phosphorylation of the serine hydroxyl is very rapid (9), presumably utilizing the acylation mechanism of substrates. Inactivation by BrPin would prevent this as it prevents acylation by substrates. Aging of DFP-inactivated enzyme removes an isopropyl group and this may allow entrance of [^{14}C]BrPin; also rotation about the serine-phosphate bond may bring the anionic phosphate out into the medium. Further, the trimethyl cavity is

not restricted in size to that of the β-substituents in AcCh or DMBAc. It accommodates trimethylsilyl (16) and N,N-diethyl-N-n-butylammonio (53) substituents in reactive substrates. The size of the latter indicates that BrPin may enter despite the presence of an isopropyl group. The second ^{14}C per unit inactivated does not enter the domain extending from the trimethyl site through the peripheral site occupied by the second cationic group of decamethonium, since the latter, like TAP, excludes only one ^{14}C (27).

The displacement, on inactivation by BrPin or [^{14}C]BrPin, of one peptide in the HPLC optical density profile and concentration of the ^{14}C on one residue in that displaced peptide, greatly diminished in protection by TAP, after essentially complete conversion of the labeled enzyme to peptides and elution of readioactivity from the HPLC column, assure that BrPin inactivates with high specificity. A low level of radioactivity eluting throughout the HPLC profile, little affected by reaction in the presence of TAP, arises in part from displacement of label by the conditions of the chromatography, and in part from non-specific reaction of [^{14}C]BrPin. The instability of S-pinacolonylcysteine in solution was observed during its synthesis and NMR spectrum determination. Additional, nonlocalized reaction with BrPin or [^{14}C]BrPin is reflected in incorporation of up to two ^{14}C per enzyme unit inactivated (27). Release of radioactivity after treatment with OPA at cycle 3, which prevents all subsequent cleavage of residues, similarly indicates that low level radioactivity eluting at each cycle arises from action of the sequencing reagents and conditions. Treatment with OPA at cycle 8, where the Ala-222 peptide has a proline residue which does not react with OPA, and the subsequent release of high radioactivity at cycle 10, demonstrate that it is this peptide which bears the label. Sequencing of the N-terminal portion of the enzyme, and of this peptide, showed no deviation from the deduced structure of T. californica (44), allowing that to be used. This identified the labeled residue as Cys-231, previously reported present in its reduced form in T. californica (54). This was confirmed by the preparation and use of S-pinacolonylcysteine. The ^{14}C-sequencing profiles indicate that some labeling may also occur at low level at Ser-226 and Ser-228. Their hydroxyls, 4.3Å and 5.6Å distant from His-440 ND1 and NE2 respectively, may facilitate binding of cationic compounds related to AcCh in the active site.

The X-ray diffraction study (26) places S of Cys-231 at 8.28, 8.11 and 7.33 Å distant from hydroxyl-O of Ser-200, imidazole-1-C of His-440 and carboxyl-O of Glu-327, respectively. Thus a pinacolonyl group on S of Cys-231 can well prevent access of substrates or DFP to the esteratic site (27). Cysteine does not have a function in catalysis by AcChE, and our early kinetic studies, which led us to BrPin, were carried out on AcChE from Electrophorus which has no free sulfhydryl. BrPin, a general

alkylating agent, must react with a different nucleophile in the active site of that AcChE. Its reaction with Cys-231 in enzyme from T. nobiliana is supported by the inactivation of this enzyme by the sulfhydryl-specific reagent, 2-PDS, which does not inactivate the enzyme from Electrophorus. That preinactivation by 2-PDS largely prevents subsequent reaction with [^{14}C]BrPin and excludes it from the Ala-222 peptide supports reaction of both reagents at Cys-231.

2-PDS binds competitively and reacts with Cys-231, forming the mixed disulfide with rate constant, k_2/K_i = 5.0 x 10^{-4} s^{-1}/39 x 10^{-6} M = 13 ± 1.5 M^{-1} s^{-1}. This may be compared with k_2 = 1.8 x 10^{-4} s^{-1} in reaction of BrPin with Cys 231 in AcChE from T. nobiliana, and 1.0 x 10^{-4} s^{-1} in reaction with a different nucleophile in its complex with Electrophorus (17). The value of K_i for 2-PDS observed by irreversible inhibition, 28 μM (Fig. 8 and eq. 4) is slightly more favorable than that determined more directly (Fig. 6), possibly because of some inactivation by this very reactive reagent during the manipulations. The binding of 2-PDS appears to involve interaction with Cys-231, since its interaction with enzyme from Electrophorus is weaker and largely noncompetitive (Fig. 6) and comparable to binding of simple benzene derivatives to that enzyme (24). In addition, binding of 2-PDS does not lead to inactivation of AcChE of Electrophorus. BrPin, on the other hand, binds competitively and similarly to both, K_i = 0.2 mM, and inactivates both with lower rate constants, 1.0 and 0.5 M^{-1} s^{-1} respectively.

While inactivation by BrPin led to displacement of the Ala-222 peptide in the HPLC profile, inactivation by 2-PDS did not. In preparation for HPLC, the introduced disulfide is reduced by DTT, like those naturally present in untreated enzyme, and no effect of this inactivation by 2-PDS remains in digested protein. The inactivation by 2-PDS, like that by BrPin, is retarded by TAP, consistent with presence of the cysteine and the action of these compounds at the active site. The protection against 2-PDS appears less effective than would be calculated from their K_i values, indicating that, binding at the trimethyl site, TAP hinders but may not completely exclude 2-PDS from the contiguous aryl site. Other sulfhydryl reagents examined in this study were less effective than 2-PDS. Aromatic side chains in proteins, like analogous simple aromatic compounds, interact more favorably with electrophilic species, and the carboxylate groups in DTNB would lead to weaker binding; IAm has neither aliphatic nor aromatic binding groups and is ineffective. Excess negative charges on the enzyme of isoelectric point ~5 would also repel carboxylate containing DTNB and IAc, although the latter is used at high concentration to alkylate sulfhydryl in denatured protein. Weak or absent effects of DTNB, IAm and IAc on AcChE from T. californica, similar to those on enzyme from T. nobiliana, have been reported (55).

There are other somewhat conflicting reports on effects of sulfhydryl reagents on the activity of AcChE. p-Chloromercuriphenylsulfonate inactivates AcChE from T. californica about as rapidly as 2-PDS inactivates the enzyme from T. nobiliana, and it was suggested that reaction occurs at Cys-231 (55). However, the failure of certain quaternary substituted maleimide sulfhydryl reagents to be more effective inactivators than uncharged analogues, and to be even much less effective, led to the further suggestion that the Cys-231 sulfhydryl is distant from the "anionic site," and thus not in the active site. Our results, consistent with the recent X-ray results, establish the proximity of Cys-231 to the catalytic triad. In contrast to the inactivating reactions at Cys-231 which prevent subsequent reactions with serine phosphorylating agents, S-mercuri-N-dansylcysteine has been reported to react quantitatively with AcChE from T. californica without affecting its activity, as indicated by subsequent reaction with M7CQ at the active-site serine (56, 57). The reason for this is not clear. The flexibility of the reagent and its carboxylate may draw it into the aqueous phase, leaving the active site accessible. We find that prior inactivation by BrPin or 2-PDS prevents subsequent reaction with M7CQ.

While these reactions at Cys-231 are interesting, allowing study of factors affecting certain reactions in the active site, BrPin is not sulfhydryl-specific, and it inactivates AcChE from Electrophorus efficiently in the absence of a free sulfhydryl. BrPin is a reactive active-site-directed alkylating agent, and its binding to AcChE from both T. nobiliana and Electrophorus is competitive and similar in the two. It acts from the trimethyl cavity at which the β-substituent of AcCh binds, and it also inactivates AcChE from human erythrocytes, which, like that from Electrophorus, is unaffected by 2-PDS, apparently also lacking a sulfhydryl in the active site. BrPin inactivates the enzyme from Electrophorus slightly less rapidly than that from T. nobiliana, alkylating a different nucleophile. Thus, studies with [^{14}C]BrPin on these enzymes will label other residues in the active site. BrPin did not inactivate human serum or horse serum BuChE, and 2-PDS also did not inactivate them. The linear structure of human serum BuChE has 53.8% homology with AcChE from T. californica (58), and essentially the same three intraunit disulfide bonds, cysteines 65-92, 252-263, 400-519 in the BuChE (58), and 67-94, 254-265, 402-521 in the AcChE (54), indicating similar folding patterns. The failure of BrPin to inactivate from the trimethyl binding sites, which are presumably present in these cholinesterases, may arise from absence of an appropriate nucleophile or, more likely, its displacement in the active site.

B. Inactivation by Phenacyl Bromide

PhABr is under consideration as a labeling agent acting from the aromatic area of the active site. Radioactive acetophenone

is commercially available, and microscale bromination of unlabeled material has been successful. AcChE from T. nobiliana appears to be inactivated about 100x more rapidly than enzyme from Electrophorus, but the difference may be less when affinity purified Electrophorus enzyme is studied. Conditions for protection by PTA have been worked out, and specific labeling and identification of labeled amino acids should be feasible. Other reversible inhibitors (Table III) seem less suitable for such labeling. NMPy, DEBAP and TAP may be of interest in studies of long-period inactivation while PTA may be informative on both long and short inactivations. Notably, tetramethylammonium ion (TeMA) offers little if any protection to Electrophorus enzyme, indicating that this small molecule, with no carbonyl and no aromatic groups, may occupy the trimethyl site while PhABr binds at the aryl site and sends its bromoacetyl groups toward the esteratic site. However, prior inactivation by PhABr of enzyme from T. nobiliana excluded 71% of ^{14}C on subsequent treatment with [^{14}C]BrPin as compared with a control. Both reagents may react with Cys-231 in this enzyme, and it will be of interest to make such studies, as well as studies with [^{14}C]BrPin-TAP systems, with purified enzyme from Electrophorus.

It may be noted (i) that PhABr and BrPin inactivate AcChE from human erythrocyte while 2-PDS does not, and (ii) PhABr inactivates human BuChE specifically, with protection by TAP, while BrPin and 2-PDS do not inactivate this enzyme.

An alternative course to radioactive labeling by bromoketones, inactivation by cold reagent followed by reduction of the carbonyl by [^{3}H]BH_4^-, was examined briefly with enzyme from T. nobiliana. Two difficulties became apparent in studies with BrPin and PhABr: (i) introduction of excessive nonspecific label and (ii) displacement of the inactivating ketonic groups from its enzyme-nucleophile bond by the strongly nucleophilic hydride. Reaction with initially radioactive α-haloketone is the preferred course for these studies.

C. Inactivation by Methyl Benzenesulfonate

Methyl benzenesulfonate (MBS), a third potential radioactive labeling agent, acting from the aryl site has been synthesized on a microscale, and protection against it has been studied, (Table IV). Strong protection against it by NMAcr, 3-AcPy and 2-DMAPy indicate that the labeled active-site residue can be identified. MAcr and 3-AcPy, not yet studied as protecting agents against PhABr, merit such study. PTA, which strongly protected against PhABr, did not protect against MBS; on the other hand, 2-DMAPy offered little protection against PhABr. Protection against the two inactivators is summarized in Table XI. A clear pattern cannot be derived. Occupation of the trimethyl site alone is not sufficient to protect against an aromatic inhibitor, while it does protect against BrPin. On the other hand, presence of an

aryl group is not sufficient to have an effect. The aryl domain is large; two inactivators may act from different parts and the various inhibitors may bind at different parts. The inactivators may label different residues, and identification of them will be informative.

While occupation by an inhibitor of the trimethyl site alone did not prevent reaction with MBS, introduction of the methyl group from the inactivator, in inactivation of AcChE from T. nobiliana, led, on subsequent treatment with [^{14}C]BrPin, to exclusion of 70% of ^{14}C as compared with a control. A similar result was obtained on prior inactivation by PhABr. Since [^{14}C]BrPin labels Cys-231 of this enzyme, it may be that MBS and PhABr do also. If MBS does, then introduction of the small methyl group, like that of the large pinacolonyl group, prevents access of substrates and of BrPin to the trimethyl site. It will be of interest to examine the sites of labeling, with the radioactive aromatic inhibitors, of AcChE from T. nobiliana, and that from Electrophorus which does not have a cysteine in the active site.

D. Binding to AcChE-Substrate Complex in Noncompetitive Inhibition (20)

Calculation of the [EA]/[E·S] ratios in hydrolysis of these substrates depends on k_2 values, and these depend on the k_2/k_3 ratio for AcCh. An early value of k_2/k_3 ratio = 6 (45) is more relevant to the present study, in terms of the enzyme form and the ionic strengths used, but the uncertainty in the value was large. A smaller value of 2.1 for the ratio applies to somewhat less relevant conditions (46), but with either value little EA and much E·S are present in hydrolysis of the slower substrates.

In an attempt to assess whether the size of TeMA and/or the NH^+ group of TrMA lead to the more favorable $K_{i(nonc)}$ value of the latter in hydrolysis of AcCh, we examined, initially, inhibition by their isosteric NH^+- containing isomers, tert-butyl- and isopropylammonium ions, IA and IB (Table VI). Their somewhat stronger $K_{i(com)}$ values than their isomers, IIA and IIB, may reflect greater interaction of their exposed positive charges than that of the insulated ones of IIA and IIB to the multiple negative surface charges (15). Surprisingly, both IA and IB showed opposite effects to that of TrMA, generally stronger noncompetitive inhibition of the slower substrates than of AcCh, i.e., under conditions of high E·S and low EA content, indicating the formation of E·S·I' rather than EA·I complexes as the major source of their noncompetitive inhibition. The branched hydrocarbon structures of IA and IB are essential for their binding, since the linear n-propyl- and n-hexylammonium ions have very poor $K_{i(nonc)}$ values in hydrolysis of AcCh, ~55 mM, and also less favorable $K_{i(com)}$ values than IA and IB (59).

Study of quaternary and tertiary ammonium ions more closely related to AcCh in structure revealed a marked difference between the two groups. The generally more favorable $K_{i(nonc)}$ values in inhibition of the slower substrates by the quaternary cations, TeMA, IIA, choline, IIIA, choline ethyl ether, IVA, and acetamidocholine, VA, the latter two nearly isosteric with AcCh, lead to the conclusion that their noncompetitive inhibition arises from E·S·I' and not EA·I complexes. Thus E·S·I' complexes, hitherto considered as formed by cations with large organic substituents (60), possibly binding in nonpolar regions (53), are also formed from these quaternary AcCh analogues with small trimethylammonio groups. The tertiary compound, VB, also forms an E·S·I' complex, by this criterion, possibly arising from amide-amide hydrogen bonding interactions, more accessible from a peripheral site than from the active site.

Generally less favorable $K_{i(nonc)}$ values for the tertiary cations, IIB, IIIB and IVB, with substrates of lower reactivity than with AcCh, are consistent with but do not require their preferred binding to EA, forming EA·I complexes, as has been proposed and is commonly accepted (46,18,61,62). Their stronger effects on the hydrolysis of AcCh indicate that they decrease deacylation rates more than those of acylation, but do not distinguish whether they do so via EA·I or EA·I' complexes. (I′ or S′ indicates I or S bound to the peripheral site.)

Stronger inhibition by tetra ethylammonium ion, VI, of the slower uncharged substrates than of AcCh, is also to be attributed to formation of E·S·I' complexes, and its favorable $K_{i(com)}$ may indicate similarly strong binding to the active site of the free enzyme. Favorable $K_{i(com)}$ and $K_{i(nonc)}$ values of the still larger tetra-n-propylammonium ion, VIIA, also reflect E·I and E·S·I' complexes. These quaternary cations with larger N-alkyl substituents would bind even less well in the decreased volume of EA as compared with E, than those, above, with trimethylammonio substituents, and the interpretation of their behavior based on the relative degrees of blocking of deacylation of their EA·I complexes is illogical. The favorable $K_{i(nonc)}$ values of the tertiary tri-n-propylammonium ion, VIIB, with AcCh and DMBAc, as compared with the $K_{i(com)}$ value, would be interpreted in the same way as those due to E·S·I' complexes. The poor $K_{i(nonc)}$ values of VIIB and IB in hydrolysis of DEBAAc would reflect need to compete with peripheral binding by the substrate itself.

The strong binding and low k_{cat} of DEBAAc reflect nonproductive binding. Its $K_{i(com)}$ value, 2.2 mM, as inhibitor of hydrolysis of AcCh (XI, Table VI) may correspond to its true binding to the free enzyme in its own hydrolysis, rather than the low observed K_m value, 0.20 mM. Its more favorable $K_{i(nonc)}$ value does not reflect stronger binding of its large, quaternary, β-substituent in the restricted volume of EA than to free enzyme; it arises from binding to a peripheral site, like that of other

cations with large alkyl substituents. Thus, DEBAAc acts as its own noncompetitive inhibitor, via formation of E·S·S', and decreases its acylation rate. Its low apparent K_m value derives from small increase in rate with increasing concentration, an artifact of its own noncompetitive inhibition, via peripheral binding, as it acts in hydrolysis of AcCh. While the value in one system may not be transferred to another with precision, use of the $K_{i(com)}$ value in place of the observed K_m, and the k_{cat} value, 2.3×10^3 s^{-1}, leads to $k_{cat}/K_m = 1 \times 10^6$ $M^{-1}s^{-1}$ rather than the higher apparently nominal value of 1×10^7 $M^{-1}s^{-1}$ (Table V).

The ketone analogue of AcCh, TAP, VIIIA, shows no noncompetitive inhibition. Its very strong competitive binding, $K_{i(com)}$ = 0.035 mM, arises from synergistic interactions in the trimethyl and esteratic sites, as the serine hydroxyl is attracted to the carbonyl-carbon. Such synergism would not be available in a peripheral site, and a $K_{i(nonc)}$ value similar to those of the other quaternary trimethyl compounds would not be observed in the concentrations that can be used in study of this strongly binding competitive inhibitor. The lower homologue, VIIIB, a weaker competitive inhibitor, $K_{i(com)}$ = 1 mM, was studied at higher concentration, and noncompetitive inhibition of AcChE and DMBAc was observed. The larger, quaternary, triethyl homologue, IXA, with less favorable $K_{i(com)}$, probably due to diminished synergism in active-site binding, was studied at higher concentration and does show noncompetitive inhibition of hydrolysis of AcCh. In accord with the large substituent size, this may also be attributed to peripheral binding via an E·S·I' complex. A similar interpretation is given to the stronger noncompetitive inhibition of AcCh and DMBAc by the bulkier 5-N,N-diethyl-N-n-butylammonio-2-pentanone, X. The less favorable $K_{i(nonc)}$ values of the tertiary ammonio ketone, IXB, may reflect a decreased tendency of trisubstituted ions to form E·I' complexes.

An E·S·I' complex may be formed more readily with one cationic ligand than with two. This may account for generally favorable $K_{i(nonc)}$ values in hydrolysis of uncharged DMBAc, including those with tertiary cation, IIB, IIIB and VB, and in part for less favorable $K_{i(nonc)}$ values for DEBAAc than for DMBAc. More important, added inhibitors must compete for the peripheral site with DEBAAc, acting quite effectively as its own noncompetitive inhibitor, and this would result in poor observed $K_{i(nonc)}$ values for them.

Varied inhibitors, some closely related in structure to AcCh, bind peripherally, forming E·I' and then E·S·I' complexes. These are also formed from E·S, to a greater extent from the less reactive substrates. The E·S·I' complexes are conformationally affected species (53,63) and the inhibitor decreases rates of acylation. This is the rate-limiting step in hydrolysis of less reactive substrates, and favorable $K_{i(nonc)}$ values are found.

Hydrolysis of AcCh would be less affected by peripheral inhibitor binding, as reflected in the $K_{i(nonc)}$ values, because of higher [EA]/[E·S] ratio, and rate limiting deacylation. But E·I', E·S·I', and EA·I' complexes are formed in hydrolysis of AcCh, and have their effects. AcCh itself, like the structurally related trimethylammonio quaternary compounds (Table VI), also binds peripherally, forming E·S', E·S·S' and EA·S' complexes. Like DEBAAc, but less effectively, it acts in this way as its own noncompetitive inhibitor. Inhibition by substrates is characteristic of AcChE: AcCh and homologues in which one N-methyl was replaced by an ethyl, n-propyl or n-butyl, and another set in which two N-methyls were replaced by ethyl and the third was varied from methyl through n-butyl, leading to DEBAAc, all showed bell-shaped curves of hydrolysis rate vs. concentration (64). Steady-state kinetic studies do not allow distinction between binding to acetylenzyme, EA·S, and to enzyme-substrate complex, E·S·S', as the source of inhibition by substrates (65). However, the behavior of the above sets of substrates (64) and the results of the present study, including inhibitors isosteric with or otherwise closely related to AcCh, indicate that formation of E·S·I' complex generally occurs, and that inhibition by substrate in hydrolysis of AcCh occurs via E·S·S' and EA·S', and not EA·S. Preferred binding to the trimethyl site of EA, if it occurs, would be a rather special case, found with some tertiary dimethylammonio ions, and these are not satisfactory models for AcCh. Thus, binding of cationic substrates to a peripheral site of AcChE should be included as of general occurrence.

Binding of substrate to a peripheral site performs an essential function. An initial inhibition period is required to allow AcCh to act on the receptor before it is hydrolyzed and its action is terminated by the very efficient, closely associated enzyme. To this end a peripheral, regulatory, binding site evolved, at which an endogenous regulator (and, later, pharmacological agents) could bind. In this case, the neurotransmitter can act as the regulator of its own destruction (66). Released initially in high concentration in the restricted volume of the synapse, it leads to the response of the receptor while it strongly retards its own hydrolysis. Then, as its concentration slowly decreases, its hydrolysis rate increases sharply to the peak of an unsymmetrical bell-shaped curve, and then decreases to depletion.

E. Pyridine Derivatives as Inhibitors and Inactivating Agents

Despite its greater water solubility, pyridine binds more strongly than benzene, $K_{i(com)}$ = 8.1 mM vs. 23 mM (24). This may arise from its greater electronegativity and thus greater charge transfer interaction with electron-donating aromatic groups in the binding site (24, 26). Increase in binding by electron-

attracting substituents is similarly accounted for, and increase due to aliphatic substituents is consistant with the hydrocarbon character of the binding site (16). Increase in binding due to electron-donating methoxy and amino groups is in contrast to their effects on benzene, where they reduce binding to $K_{i(com)}$ 50 mM. Substituted benzenes show a regular dependence of binding on Hammett sigma values, ρ = +2 (24). In pyridines, electron-donating substituents increase electron density on the heterocyclic N, increasing its basicity and raising the concentration of protonated - and more strongly binding - species at a given pH. The pyridine-N-oxides, with binding similar to that of the pyridines, also differ from their tertiary aliphatic analogues, where conversion of the amine, present as ammonium ion, to the oxide essentially destroys binding (16).

The pyridines, like substituted benzenes, show both competitive and mixed inhibition, and no clear pattern affecting these properties seems to emerge. The benzenes appear to show somewhat more noncompetitive and thus mixed inhibition than the pyridines, indicating that the latter, perhaps cationic as they bind, exclude cationic AcCh from the binding cavity, while benzenes, uncharged, may to a degree bind along with substrates in the cavity and affect reactivity. The brief study of noncompetitive inhibition by benzene and pyridine derivatives as a function of substrate reactivity (Table VIII) indicates little difference in comparison of AcCh and DMBAc for four of the compounds, 3-AcPy, NMPy, acetophenone, and nitrobenzene, and more effective noncompetitive inhibition for 2-DMAPy and 3-tert-butylphenol with the slower substrate, DMBAc. These latter two inhibitors and the slower substrate have substituents which may bind in the trimethyl site and would not be expected to bind simultaneously in the active site. A second site seems to be involved in their binding.

The results described above were with AcChE from Electrophorus. The few pyridines studied with AcChE from T. nobiliana (Table IX), showed similar competitive or mixed inhibition with each. 2-PDS, interacting with Cys-231 of T. nobiliana, shows markedly different behavior with Electrophorus (Fig. 6).

Most notable is the very strong binding of the dimethylamino pyridines, similar to that of N-methylpyridinium ions, (Table VII). The DMAPys exist in solution in equilibria of neutral and cationic forms, and the neutral forms allow efficient permeation through membranes and then, protonated, they can bind strongly. Thus, for example, a dimethylamino pyridaldoxime may prove superior to the N-methylpyridinium aldoxime as a reactivating agent. Similarly, if strongly binding reversible inhibitors are desired for other medicinal purposes, they may be found in this class of compounds.

3-Bromoacetylpyridine, 3-BAPy, showed similar effectiveness to PhABr in inactivating AcChE from Electrophorus. However, while PTA offered complete protection against PhABr over 50% inactivation in its absence, it offered only partial protection against 3-BAPy, as did NMPy and 3-AcPy. In general, the aromatic reversible inhibitors showed similar patterns in protecting against PhABr and BAPy, while purely aliphatic compounds, where examined, TeMA, TAP, DeMe, and tubocurarine showed little protection against these aromatic inactivators. The aliphatic active-site-directed reversible inhibitors protect strongly against BrPin, and exclude the aliphatic substrates. They may fit into the active site with the aromatic inactivatiors and not protect. The aromatic inactivatiors are effective against the aliphatic substrate, AcCh, either by preventing its access or by alkylating at the esterolytic site.

The preceding studies with aromatic inactivators were carried on with crude, commercially available, AcChE from Electrophorus. We have now isolated AcChE from this electric organ, purified it by affinity chromatography, inactivated it with [^{3}H]DFP, isolated the labeled peptide and sequenced it over 21 cycles and find the labeled peptide to be identical, with two exceptions, to that from T. californica, starting at Thr-193, with the label at the 8th amino acid, consistent with Ser-200. We will confirm this by further isolation and purification. We will seek to identify the active-site amino acid residues in both T. nobiliana and Electrophorus labeled by [^{14}C]BrPin and the radioactive aromatic inactivators, as we prepare them.

Table I

Yields of PTH-Amino Acids from Sequence Analysis of Fractions Containing the Ala-222 Peptide[a].

Cycle #	Amino acid	PTH-amino acids (pmol) Fraction 64 -TAP	Fraction 64 +TAP	Fraction 76[b] -TAP	Fraction 76[b] +TAP	Fraction 76[b] -TAP OPA #7[c]	Fraction 76[b] -TAP OPA#3[c]
1	Ala	53	102	106	50	95	97
2	Ile	39	99	80	38	61	70
3	Leu	35	93				
4	Glu	31	76				
5	Ser	NQ	NQ[d]				
6	Gly	20	47	35	20	32	<1
7	Ser	NQ	NQ				
8	Pro	20	46	37	16	26	<0.6
9	Asn	15	39				
10	CM-Cys	NQ	NQ				
11	Pro	12	29	6.3	4.4	7.1	
12	Trp	NQ	NQ				
13	Ala	8.0	23	6.3	4.2	4.2	
14	Ser	NQ	NQ				
15	Val	7.2	16				
16	Ser	NQ	NQ				
17	Val	2.4	7.7				
18	Ala	4.3	11.5				
Initial yield (pmol)[e]		58 ± 2	125 ± 5	127 ± 10	59 ± 1		
Repetitive yield, (%) [e]		86 ± 1	88 ± 1	82 ± 2	83 ± 2		

[a] Fractions 64 and 76 from the trypsin digests of AcChE inactivated by [^{14}C]BrPin in the absence or presence of TAP (Fig. 3) were subjected to sequence analysis: 20% of each sample was sequenced, and a portion (40%) of each cycle was used to detect PTH-amino acids. The values shown are the observed release.

[b] As described in Methods and Results, Fraction 76 contained 4-5 peptides in addition to the Ala-222 peptide. Only amino acids unique to the Ala-222 peptide were used to estimate mass amounts.

[c] Cycle No. after which OPA treatment was carried out.

[d] Not quantified.

[e] Calculated as described in methods.

Table II

Effects of Sulfhydryl Reagents on AcChE from T. nobiliana.

AcChE	Inactivator		Incub.	Inactiv.
μM	Cpd.	μM	t. hr.	%
0.80	---	--	18	0
0.80	2-PDS	33	18	66[a]
0.04	---	--	1.7	6
0.04	2-PDS	22	1.7	93
0.04	DTNB	22	1.7	18
0.017	---	--	22	40
0.017	IAm	230	22	60[b]
0.017	IAc	230	22	40

[a]Reduced to 0% by 14 mM TAP.

[b]Reduced to 30% by 12 mM TAP.

Table III

Inhibition of Inactivation by 1.6 mM PhABr ($1xK_i$) of AcChE (Electrophorus, Sigma, 4-9 nM) pH 7.8, 0.18 M NaCl, 0.5% CH_3CN, 25°C.

Incubation hr.	Inactivation[a] %	Inhibitor Compound	Inhibitor mM	Inactivation, % + Inhibitor
6	55	PTA	0.9[b]	0
24	100	PTA	0.9	35
6	45	NMPy	1.6[b]	28
24	100	NMPy	1.6	52
6	45	DEBAP	1.8[b]	28
24	100	DEBAP	1.8	58
6	50	TAP	0.35[b]	34
24	100	TAP	0.35	51
6	34	2-DMAPy	1.0[c]	27
24	78	2-DMAPy	1.0	64
5	51	TMAAPh	0.005[b]	29
24	100	TMAAPh	0.005	100
6	39	TeMA	29[b]	33
24	100	TeMA	29	100
0.5[d]	100	PTA	0.24[b]	100
0.17[d,•]	61	PTA	0.24	11
0.33[d,•]	88	PTA	0.24	53
0.67[d,•]	97	PTA	0.24	80[f]

[a] Inactivation by PhABr in absence of inhibitor
[b] 10 x $K_{i(com)}$
[c] 13 x $K_{i(com)}$.
[d] AcChE from T. nobiliana, 10 nM
[•] 0.25 mM PhABr, 17 nM AcChE, T. nobiliana
[f] (Control lost 25% activity)

Table IV Inactivation of AcChE from Electrophorus (Sigma, 2-6 nM) by 1.5 mM Methyl Benzenesulfonate (MBS); Protection by Reversible Inhibitors, pH 7.8, 0.18 M NaCl.

Incubat. min.	Inactiv. %	Inhibitor Compound	Inhibitor mM[a]	Inact. % + Inhibitor
60	55	MACr	0.005	0
90	74	MACr	0.005	59
105	73	3-AcPy	21.	0
1440	100	3-AcPy	21.	71
120	76	2-DMAPy	1.0	0
210	90	2-DMAPy	1.0	10
2880	100	2-DMAPy	1.0	94
60	31	NMPy	1.6	13
90	56	NMPy	1.6	42
150	82	NMPy	1.6	65
90	80	TAP	0.35	80
120	90	TAP	0.35	90
45[b]	45	PTA	0.9	45
80	78	PTA	0.9	80
90	88	PTA	0.9	88
120	100	PTA	0.9	100
120	90	TMAAPh	0.035	90
60	50	propid.[c]	0.41	25
90	75	propid.	0.41	60
120	84	propid.	0.41	84

[a] 10-15 x $K_{i(com)}$ [b] 3 mM MBS [c] propidium

TABLE V

Ratio of [EA] to [E·S] in Hydrolysis of $X\text{-}CH_2CH_2OCOCH_3$ by AcChE (pH 7.8, 25°C, 0.18 M NaCl).

X-	$10^{-2} \times k_{cat}$ s^{-1} [a]	K_m mM	$10^{-4} \times k_{cat}/K_m$ $M^{-1} s^{-1}$	$10^{-2} \times k_2$ s^{-1} [b]	[EA]/[E·S] k_2/k_3
$(CH_3)_3N^+$- Cl^-	160	0.27 (±0.05)	5900	1100 (500)[c]	5.8 (2.1)[c]
[d] $(CH_3)_3N^+$- Cl^-	90 (±11)	0.22 (±0.08)	3400	175 (130)	0.92 (0.54)
$(CH_3)_3C$-	77 (±19)	1.4 (±0.2)	550	130 (120)	0.68 (0.50)
CH_3CH_2-	26 (±10)	13 (± 2)	20	30 (29)	0.16 (0.12)
[d] $CH_3N^+H_2$- Cl^-	24 (±4)	11 (±2)	21	27 (27)	0.14 (0.11)
$(C_2H_5)_2$ $n\text{-}C_4H_9N^+$- I^-	23 (±6)	0.20 (±0.07)	(1150)[e]	26 (26)	0.14 (0.11)
$CH_3S(O_2)$-	14 (±3)	7.0 (±0.09)	20	14 (14)	0.07 (0.06)

[a] Based on $k_{cat} = 1.6 \times 10^4\ s^{-1}$ for AcCh, pH 7.8; values of k_{cat} and K_m for substrates other than AcCh are averages of those obtained in studies with varied inhibitors.

[b] Based on $k_{cat} = (k_2 \times k_3)/(k_2 + k_3)$, $k_2/k_3 = 6$ for AcCh; $k_3 = 1.9 \times 10^4\ s^{-1}$

[c] Figures in parentheses are based on $k_2/k_3 = 2.1$ for AcCh; $k_3 = 2.4 \times 10^4\ s^{-1}$

[d] At pH 6.5

[e] Nominal value, reduced to 100, based on $K_m = K_{i(com)} = 2.2$ mM (Table VI), for this substrate used as inhibitor for acetylcholinesterase

TABLE VI

Noncompetitive components, $K_{i(noncomp)}$, mM, in inhibition of hydrolysis by AcChE of X-$CH_2CH_2OCOCH_3$ (pH 7.8, 25° C, 0.18 M NaCl)

Inhibitor		Substrate, X-, $K_{i(noncomp)}$, mM						
		$(CH_3)_3N^+$-	$(CH_3)_3C$-	CH_3CH_2-	$CH_3N^+H_2$-	$(C_2H_5)_2(n\text{-}C_4H_9)N^+$-	$CH_3S(O_2)$-	$K_{i(noncomp)}$ Av. mM
IA	$(CH_3)_3CNH_3^+$ Cl^-	6.9 (±2.0)	1.7 (±0.3)			1.5 (±0.4)	1.5(±0.2)	0.7 (±0.2)
IB	$(CH_3)_2CHNH_3^+$ Cl^-	23 (±2)	1.2 (±0.3)	5.9(±2.1)		19 (±2)	5.8(±0.7)	1.9 (±0.4)
IIA	$(CH_3)_4N^+$ Cl^-	7.5 (±0.5)	5.2 (±1.3)	3.4(±1.0)		11 (±1.4)	2.8(±0.6)	2.6 (±0.4)
IIA[a]	$(CH_3)_4N^+$ Cl^-	14 (±3)			5.1(±1.2)			2.9 (±1.0)
IIB	$(CH_3)_3NH^+$ Cl^-	3.3 (±0.3)	1.2 (±0.1)			6.1 (±0.6)	22 (±7)	2.1 (±1.3)
IIB[a]	$(CH_3)_3NH^+$ Cl^-	5.8 (±0.9)			9.3(±0.8)			10.9 (±0.3)
IIIA	$(CH_3)_3N^+CH_2CH_2OH$ Cl^-	7.4 (±2.0)	1.7 (±0.4)	3.4(±1.0)		4.2 (±0.3)	2.2(±0.2)	0.9 (±0.1)
IIIA[a]	$(CH_3)_3N^+CH_2CH_2OH$ Cl^-	3.1 (±0.04)			5.8(±0.4)			1.8 (±0.6)
IIIB	$(CH_3)_3N^+HCH_2CH_2OH$ Cl^-	2.5 (±0.6)	2.8 (±0.3)	6.6(±1.6)		6.5 (±1.1)	6.7(±0.5)	3.2 (±1.6)
IIIB[a]	$(CH_3)_3N^+HCH_2CH_2OH$ Cl^-	5.8 (±1.4)			8.5(±0.8)			5.5 (±0.8)
IVA	$(CH_3)_3N^+CH_2CH_2OC_2H_5$ I^-	9.6 (±1.1)	3.9 (±1.0)	0.7(±0.2)				0.54 (±0.24)
IVB	$(CH_3)_3N^+HCH_2CH_2OC_2H_5$ Cl^-	0.26(±0.08)	0.65(±0.19)	2.1(±0.5)		1.9 (±0.2)		3.7 (±1.0)
VA	$(CH_3)_3N^+CH_2CH_2NHCOCH_3$ I^-	11 (±1.7)	2.6 (±0.3)			7.5 (±0.7)		3.3 (±0.5)
VB	$(CH_3)_3N^+HCH_2CH_2NHCOCH_3$ Cl^-	4.7 (±0.7)	1.7 (±0.1)			3.2 (±0.6)		2.6 (±1.0)
VI	$(C_2H_5)_4N^+$ Cl^-	2.1 (±0.3)	0.3 (±0.12)			2.6 (±0.1)		0.34 (±0.05)
VIIA	$(CH_3CH_2CH_2)_4N^+$ Cl^-	0.13(±0.01)	0.16(±0.01)			0.54(±0.16)		0.13 (±0.07)
VIIB	$(CH_3CH_2CH_2)_3N^+H$ Cl^-	0.32(±0.04)	0.51(±0.06)			7.9 (±1.0)		1.1 (±0.9)
VIIIA	$(CH_3)_3N^+CH_2CH_2CH_2COCH_3$ I^-	[b]	[b]	[b]			[b]	0.035(±0.02)
VIIIB	$(CH_3)_3N^+H(CH_2)_3COCH_3$ Cl^-	2.5 (±0.6)	3.6 (±0.9)					1.0 (±0.4)
IXA	$(C_2H_5)_3N^+(CH_2)_3COCH_3$ I^-	2.7 (±0.4)						0.5 (±0.1)
IXB	$(C_2H_5)_3N^+H(CH_2)_3COCH_3$ Cl^-	7.4 (±0.8)						0.4 (±0.2)
X	$(C_2H_5)_2(n\text{-}C_4H_9)N^+(CH_2)_3COCH_3$ I^-	0.30(±0.1)	0.20(±0.02)					0.19 (±0.02)
XI	$(C_2H_5)_2(n\text{-}C_4H_9)N^+CH_2CH_2OCOCH_3$ I^-	0.87(±0.14)						2.2 (±0.3)

[a] At pH 6.5.

[b] Noncompetitive inhibition not observed.

Table VII Reversible Inhibition by Pyridine Derivatives of Hydrolysis of AcCh by Electrophorus AcChE, pH 7.8[a]

Substituent	pK_a	$K_{i(com)}$ (mM)	$K_{i(nonc)}$ (mM)	$\Delta G_{(com)}$ kcal/mole
H-	5.25	8.1 ± 2.6	101 ± 38	-2.9
H-[b]	5.25	12 ± 3.0	110 ± 50	-2.6
4-$(CH_3)_3C$-	5.3	1.3 ± 0.4	47 ± 21	-3.9
4-$(CH_3)_3C$-[b]	5.3	3.7 ± 0.6	13 ± 3.0	-3.3
2-CH_2=CH-	4.98	1.4 ± 0.12	10 ± 2.5	-3.8
4-CH_2=CH-	5.62	2.0 ± 0.84	6.6 ± 1.3	-3.6
2-CH_2-CH-	3.84	1.5 ± 0.35	14 ± 1.9	-3.8
3-CH_3CO-	---	2.4 ± 0.77	2.45 ± 0.14	-3.5
3-$BrCH_2CO$-	---	2.1 ± 0.67	1.8 ± 0.37	-3.7
N-O^-	0.79	11 ± 3.7	63 ± 16.0	-2.6
4-O_2N - N-O^-	---	2.1 ± 0.3	2.7 ± 0.37	-3.7
4-O_2N-	---	2.0 ± 0.4	2.8 ± 0.35	-3.7
2-CH_3O-	---	4.0 ± 1.0	42 ± 8.0	-3.3
2-H_2N-[c]	6.86	2.2 ± 0.24	3.6 ± 0.1	-3.6
3-H_2N-	5.98	0.82 ± 0.1	Com.	-4.2
3-H_2N-[d]	5.98	10± 0.44	11 ± 1.3	-2.6
2-H_2N - 4-CH_3-[e]	7.8	0.26 ± 0.07	1.6 ± 0.15	-4.8
2-H_2N - 4-CH_3-[d,f]	7.8	1.1 ± 0.05	6.0 ± 0.7	-4.0
2-$(CH_3)_2N$-	6.99	0.17 ± 0.09	0.66 ± 0.10	-5.5
2-$(CH_3)_2N$-[d]	6.99	0.59 ± 0.04	7.4 ± 1.3	-4.4
3-$(CH_3)_2N$-	6.46	0.10 ± 0.03	0.82 ± 0.08	-5.4
3-$(CH_3)_2N$-[d]	6.46	0.79 ± 0.06	1.2 ± 0.43	-4.2
3-$(CH_3)_2N$-[g]	6.46	0.067 ± 0.02	0.25 ± 0.01	-5.6
4-$(CH_3)_2N$-[h]	9.7	0.05 ± 0.01	0.12 ± 0.01	-5.8
N-CH_3 - 3-$(CH_3)_2N$-	---	0.02 ± 0.00	0.06 ± 0.01	-6.3
N-CH_3	---	0.16 ± 0.01	0.20 ± 0.02	-5.4
3-OH-	---	Nonc.	9.3 ± 8.0	
2-$(CH_3)_2N$- 3-OH	---	3.1 ± 0.4	1.4 ± 0.2	-3.4

[a] Values at other pH's are noted; [b] At pH 8.5; weaker binding with lower protonation at higher pH; [c] 10% AH^+; [d] At pH 9; weaker binding at higher pH; [e] 50% AH^+; [f] 6% AH^+; [g] At pH 6.5; stronger binding with higher protonation at lower pH; 48% AH^+; [h] 99% AH^+; AH^+ is protonated species.

Table VIII Reversible Inhibition by Derivatives of Pyridine and Benzene of Hydrolysis of $X\text{-}CH_2CH_2OCOCH_3$, pH 7.8, 25°C, 0.18 N NaCl, by AcChE from Electrophorus.

Inhibitor	$K_{i(nonc)}$; mM	
	X- $(CH_3)_3N^+$-	X- $(CH_3)_3C$-
3-Acetylpyridine	2.5 ± 0.1	2.6 ± 0.1
N-Methylpyridinium	0.20 ± 0.02	0.17 ± 0.02
2-Dimethylaminopyridine	0.66 ± 0.10	0.11 ± 0.03
Acetophenone	3.3 ± 0.2[a]	2.5 ± 0.22
Nitrobenzene	3.0 ± 0.4[a]	2.5 ± 0.05[a]
3-Trimethylammoniophenol	4.3 ± 1.1[a] x 10^{-3}	4.4 ± 2.3[a] x 10^{-3}
3-tert-Butylphenol	0.17 ± 0.02	0.023 ± 0.002

[a] Ref. 23

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Table IX Reversible Inhibition of AcChEs from T. nobiliana and Electrophorus, mM.

Inhibitor	T. nobiliana		Electrophorus	
	$K_{i(com)}$	$K_{i(nonc)}$	$K_{i(com)}$	$K_{i(nonc)}$
4-t-BuPy	0.30	13	1.3	47
3-AcPy	12	11	2.4	2.45
2-DMAPy	0.10	0.4	0.17	0.66
PTA	0.025	0.28	0.086	0.44

Table X Inhibition of Inactivation by 3.0 mM 3-Bromoacetylpyridine of AcChE (*Electrophorus*, Sigma, 4-9 nM) pH 7.8, 0.18 M NaCl, 25°C.

Incubation hr	Inactivation[a] %	Inhibitor Compound	Inhibitor mM[b]	Inactivation, % + Inhibitor
1.75	18	NMPy	1.6	10
3.75	41	NMPy	1.6	22
5.75	48	NMPy	1.6	32
24	100	NMPy	1.6	47
3.5	37	3-AcPy	21.	20
6.2	51	3-AcPy	21.	32
24	100	3-AcPy	21.	78
3.8	38	PTA	0.9	30
6.0	52	PTA	0.9	30
2.0	27	2-DMAPy	1.0	20
5.0	45	2-DMAPy	1.0	37
6.0	50	2-DMAPy	1.0	42
3.5	36	3-TMAAPh	0.005	27
5.5	48	3-TMAAPh	0.005	41
1.5	35	TAP	0.3	28
6.0	59	TAP	0.3	54
2.8	38	TeMA	29.	29
5.5	60	TeMA	29.	49
3.0	44	DeMe[c]	1×10^{-3}	40
5.5	72	DeMe[c]	1×10^{-3}	64
4.5	45	Tuboc.[d]	2	45

[a] Inactivation by BAPy in absence of inhibitor

[b] ~ 10 x K_i

[c] ~ 100 x K_i

[d] Tubocurarine

Table XI

Inhibition of Inactivation of AcChE from Electrophorus by PhABr and MBS

	Inactivator	
Inhibitor	PhABr	MBS
PTA	strong	none
2-DMAPy	weak	strong
MAcr	n.s.[a]	strong
3-AcPy	n.s	strong
NMPy	partial	partial
TAP	partial	none
TMAAPh	partial	none
DEBAP	partial	n.s.
Propidium	n.s.	partial
TeMA	none	n.s.

[a] n.s. not studied.

Legends for the Figures

Figure 1 Reversed-phase HPLC separation of tryptic digests of *Torpedo* nobiliana AcChE. A. Enzyme (0.8 mg) was denatured, reduced and carboxymethylated as described in Methods and then digested with trypsin (5% w/w) for 24 hr at 37°C. The digest was applied to a C_{18} column and eluted at 0.5 mL/min with a linear gradient 0.1% TFA in water as solvent A and 0.075% TFA in CH_3CN as solvent B. B. Enzyme (0.8 mg) was treated for 5 hr with 1 mM BrPin, resulting in 53% inactivation and then digested with trypsin after denaturation, reduction and carboxymethylation. Separations were monitored by measuring absorbance at 219 nm, and fractions of 0.5 mL were collected. For BrPin treated enzyme, material in Fraction 64 (*) is decreased, and there is increased material in Fraction 76 (↘) that results in coalescence of peaks seen in Fractions 75 and 77 of control. Material in Fractions 64 and 75-77 were dried down and resuspended in 30% CH_3CN for sequence analyses. For each sample, 20% was applied to Biobrene treated glass fiber filters for sequence analysis.

Figure 2 Reversed-phase HPLC separation of a tryptic digest of [^{14}C]BrPin-labeled AcChE. AcChE (1.25 mg) was inactivated with 0.6 mM [^{14}C]BrPin in the absence (●) or presence (○) of 15 mM TAP. After 8.5 hr treatment, in the absence of TAP activity was reduced by 30%, while in the presence of TAP it was reduced by less than 5%. Samples were denatured, reduced and carboxymethylated and then digested with trypsin (5% w/w) for 20 hr at 37°C. Each digest was applied to a C_{18} column and eluted with a H_2O/CH_3CN gradient as in Figure 1. The separation was monitored by measuring absorbance at 219 nm (not shown, profiles as in Fig. 1) and by scintillation counting of 25 μL aliquots of 0.5 mL fractions. For each digest, Fractions 75 and 76 were combined and dried down for sequence analysis as were Fractions 63 and 64. To better quantify distribution of ^{14}C, 50% (0.25 mL) of each other fraction was counted, and ^{14}C in 0.25 mL aliquots is plotted for digests of AcChE treated with [^{14}C]BrPin in the absence (●) or presence (○) of TAP. For enzyme treated with [^{14}C]BrPin alone, 21,400 cpm were applied to the column, and 7,400 cpm (35%) were recovered in Fractions 75-76. Overall, column recovery was 80%. For AcChE inactivated in the presence of TAP, 8,000 cpm were applied to the

column, and 920 cpm (12%) were recovered in Fractions 75-76. Overall column recovery was ≈80%.

Figure 3 Release of ^{14}C upon sequence analyses of the principal [^{14}C]BrPin-labeled tryptic peptide. Material eluting in Fractions 75 and 76 (Fig. 2) was pooled, dried down and stored at -20°C until sequence analysis. Material was resuspended in 30% CH_3CN/0.1% TFA, and 90% was loaded on Biobrene-treated glass filters for sequence analysis. For AcChE inactivated in the absence of TAP (●), 5,570 cpm were loaded onto the filter, and 1,790 cpm remained after 20 cycles. For AcChE inactivated in the presence of TAP (○), 810 cpm were loaded on the filter, and 260 cpm remained after 20 cycles.

Figure 4 Reversed-phase HPLC separation of a tryptic digest of [^{14}C]BrPin labeled AcChE. AcChE (2.0 mg) was inactivated with 1.2 mM [^{14}C]BrPin in the absence (●) or presence (○) of 15 mM TAP. After 24 hr, activity in the absence of TAP was reduced by 74%, while in its presence it was reduced by 39%. After reduction and carboxymethylation, samples were digested with trypsin (2% w/w), and the digests were fractionated on a C_{18} column as in Figure 1. Distribution of ^{14}C was monitored by counting 20 μL aliquots of 0.5 mL fractions. Fraction 76 was dried down for subsequent sequence analysis. For enzyme treated with [^{14}C]BrPin alone 450,000 cpm were applied to the column and 98,500 cpm (22%) were recovered in Fraction 76, with overall column recovery of 92%. For AcChE inactivated in the presence of TAP, 285,400 cpm were applied to the column with 52,600 cpm recovered in Fraction 76.

Figure 5. Release of ^{14}C upon sequence analysis of [^{14}C]BrPin-labeled tryptic peptide; OPA blockade. Material eluting in Fraction 76 (Figure 4) was dried down and then resuspended in 30% CH_3CN/0.1% TFA for sequence analyses. Aliquots (15,850 cpm) were sequenced normally (▽) or treated with o-pthalaldehyde after the second (x) or seventh (●) cycle of Edman degradation. The cpm remaining on the filters after 22 cycles were 3,645 (▽), 8,590 (x), 4,994 (●).

Figure 6. Reciprocal plots for reversible inhibition by 2-PDS of hydrolysis of AcCh by AcChE. A. AcChE (0.03 nM) from T. nobiliana; Concentrations of 2-PDS (mM), linear regression slopes (s), and intercepts (M^{-1} s) were: (●), 0, 150, 2 4 x 10^6; (□), 0.023, 230, 2.3 x 10^6; (○), 0.046, 360, 2.4 x 10^6; (Δ), 0.084, 510, 2.3 x 10^6; (⬭), 0.172, 850, 2.6 x 10^6. Secondary plot of slope vs [2-PDS] led to slope (4.0 ± 0.1) x 10^6 M^{-1} s, intercept 154 ± 11 s, K_i = 39 ± 3 μM. B. AcChE (0.23 nM) from Electrophorus; concentrations of 2-PDS (mM), linear regression slopes (s) intercepts (M^{-1} s): (●), 0, 72, 2.8 x 10^5; (□), 0.30, 86, 3.7 x 10^5; (○), 0.60, 88, 4.4 x 10^5; (Δ), 0.90, 94, 5.0 x 10^5. Secondary plot of slopes vs [2-PDS] led to slope (2.3 ± 0.6) x 10^4 M^{-1} s, intercept 74.6 ± 3.2 s; $K_{i(com)}$ = 3.2 ± 0.4 mM; secondary plot of intercepts vs [2-PDS] led to slope (2.6 ± 0.1) x 10^8 M^{-2} s, intercept (2.8 ± 0.1) x 10^5 M^{-1} s, $K_{i(nonc)}$ = 1.2 ± 0.2 mM.

Figure 7. Irreversible inactivation of AcChE from T. nobiliana by 2-PDS. AcChE ((●), (⬭), 17 nM; (o), (□), (Δ), (x), (●), 45 nM) was incubated with 2-PDS and aliquots were diluted and examined for residual activity after the indicted times. Concentrations of 2-PDS were: (●), 2 μM; (⬭), 4 μM; (o), 10 μM; (□), 20 μM; (Δ), 40 μM; (x), 80 μM; (●), 180 μM.

Figure 8. First order rate constants, k, eq. 5, for inactivation by 2-PDS of AcChE from T. nobiliana. Values were determined from data of experiments like those in Figure 7. Slopes of linear plots of ln of residual enzymic activity as a function of time of incubation with 11 concentrations of 2-PDS (2-180 μM) are indicated, ●. The line is a hyperbolic fit of the rate constants, leading to the first-order rate constant, k_2 = (5.0 ± 0.2) x 10^{-4} s^{-1}, and K_i = 28 ± 3 μM, eq. 5.

Figure 9. First-order rate constants, k, eq. 5, for inactivation by BrPin of AcChE from T. nobiliana. Incubations were at pH 7.0, phosphate buffer, I = 0.2, 22°C. AcChE concentrations were (●), 9.6 nM and (■), 5.3 nM. BrPin concentrations were 0.020, 0.033, 0.060, 0.066, 0.080, 0.10, 0.13, 0.18, 0.50, 0.68, 0.95, 1.41, 1.54 and 2.0 mM. Aliquots (0.05 mL) were diluted 40-fold and examined for residual activity by Ellman assay after periods of

incubation. Values of k are slopes of linear plots of ln of residual activity as a function of time of incubation. The line is a hyperbolic fit of the data, leading to the first-order rate constant, k_2 = (1.8 ± 0.1) x 10^{-4} s^{-1}, K_i = 0.18 ± 0.02 mM, eq. 5.

Figure 10. Retardation by TAP of inactivation of 0.10 μM AcChE from T. nobiliana by 56 μM 2-PDS, pH 7.8, phosphate buffer, 22°C. Concentrations of TAP and rate constants of inactivation were: (●), 0, 3.0 x 10^{-4} s^{-1}; (○), 0.5 mM, 1.5 x 10^{-4} s^{-1}; (□), 1.0 mM, 0.6 x 10^{-4} s^{-1}; (Δ), 3.0 mM, 0.27 x 10^{-4} s^{-1}.

Figure 11. Reciprocal plot for reversible inhibition by phenacyl bromide (PhABr) of hydrolysis of AcCh by AcChE. AcChE (0.25 nM) from Electrophorus; concentrations of PhABr (mM), linear regression slopes(s), and intercepts (M^{-1} s^{-1}) were: (●) 0, 110, 2.6 x 10^5; (○), 0.2, 111, 3.1 x 10^5; (x), 0.6, 149, 3.3 x 10^5; (Δ), 0.8, 152, 4.3 x 10^5; (■), 1.0, 169, 4.4 x 10^5. Secondary plot of slopes vs [PhABr] led to slope 6.2 x 10^4 M^{-1} s; Intercept 106 s; $K_{i(com)}$ = 1.7 ± 0.3 mM; Secondary plot of intercepts vs. [PhABr] led to slope 1.8 x 10^{-8} M^{-2} s; Intercept 2.6 x 10^5 M^{-1} s and $K_{i(nonc)}$ = 1.4 ± 0.4 mM.

Figure 12. Reciprocal plots for reversible inhibition by methyl benzenesulfonate (MBS) of hydrolysis of AcCh by AcChE. AcChE (0.15 nM) from Electrophorus; concentrations of MBS (mM), linear regression slopes(s), and intercepts (M^{-1} s^{-1}) were; (●), 0, 49, 4.3 x 10^5; (○), 2.5 mM, 81, 4.9 x 10^5; (X), 5.0 mM, 110, 5.3 x 10^5; (Δ), 10.0 mM, 144, 6.4 x 10^5; (■), 20.0 mM, 190, 6.6 x 10^5. Secondary plot of slopes vs [MBS] led to slope 9.4 x 10^3 M^{-1} s; Intercept 55 s; $K_{i(com)}$ = 5.9 mM. Secondary plot of intercept vs [MBS] led to slope 2.1 x 10^7 M^{-2} s; intercept 4.3 x 10^5 M^{-1} s; and $K_{i(nonc)}$ = 20.6 mM.

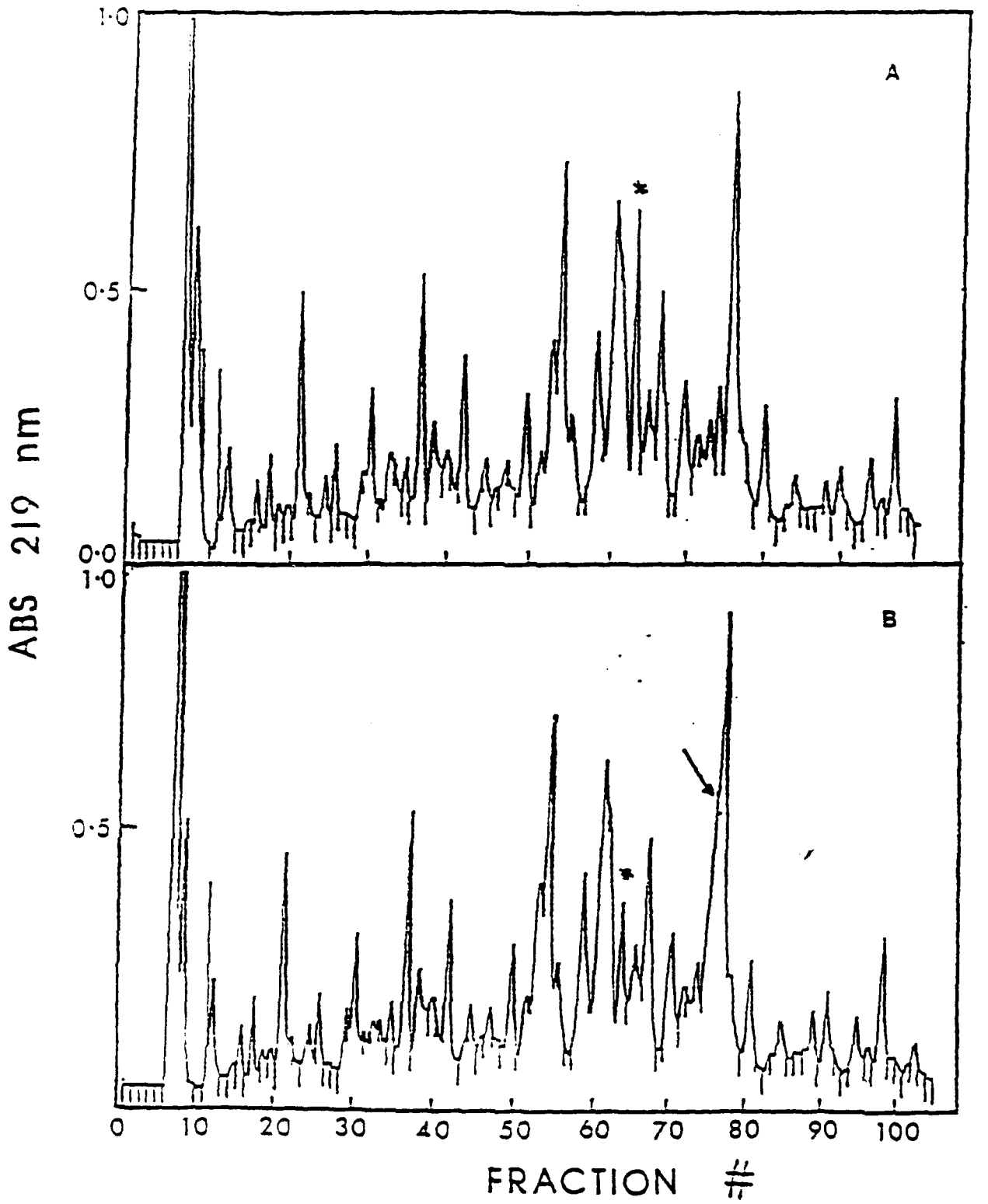


Figure 1. Reversed-phase HPLC separation of tryptic digests of Torpedo nobiliana AcChE.

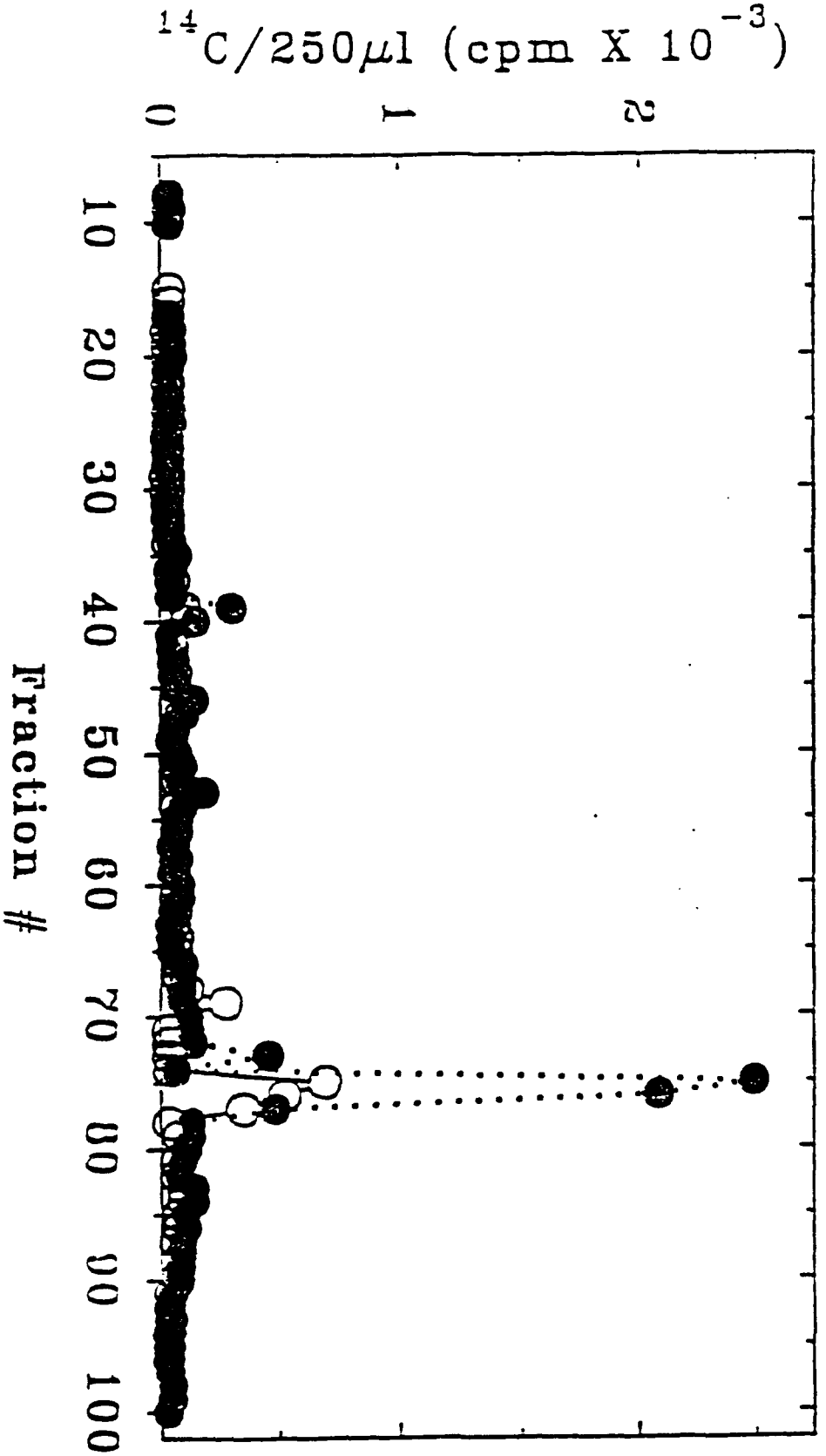


Figure 2. Reversed-phase HPLC separation of a tryptic digest of [^{14}C]BrPin-labeled AcChE.

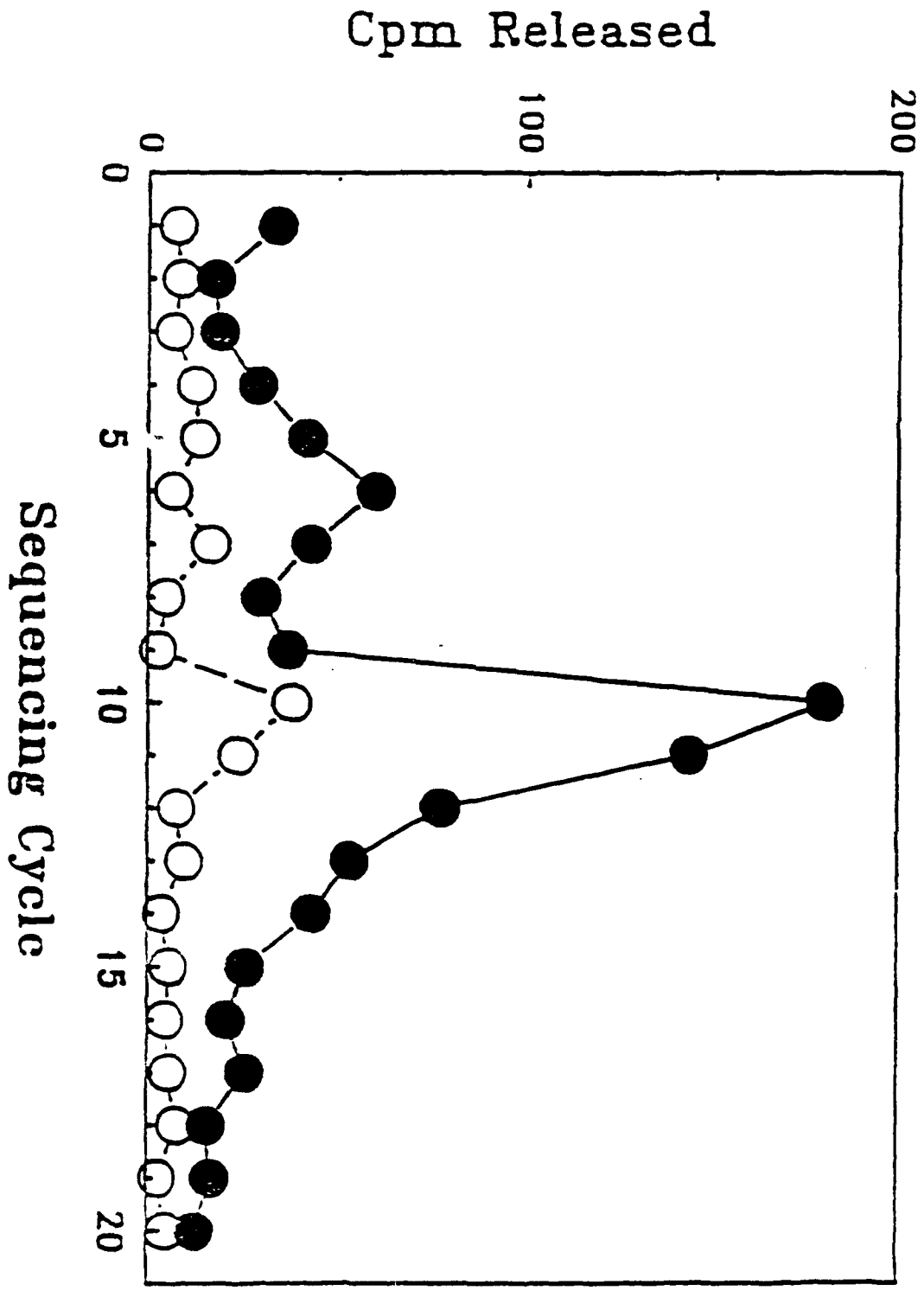


Figure 3. Release of ^{14}C upon sequence analyses of the principal [^{14}C]BrPin-labeled tryptic peptide.

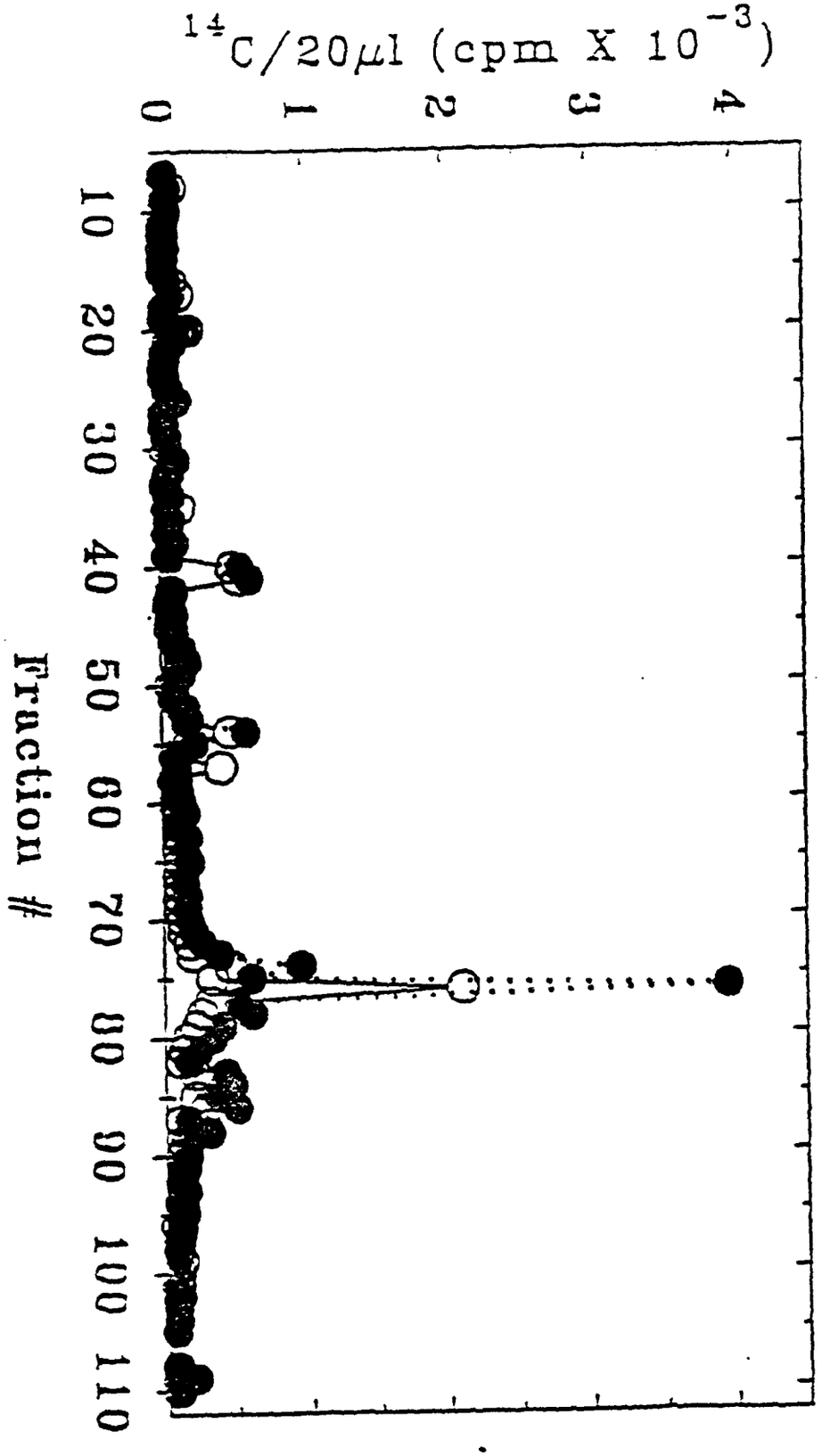


Figure 4. Reversed-phase HPLC separation of a tryptic digest of [^{14}C]BrPin-labeled AcChE.

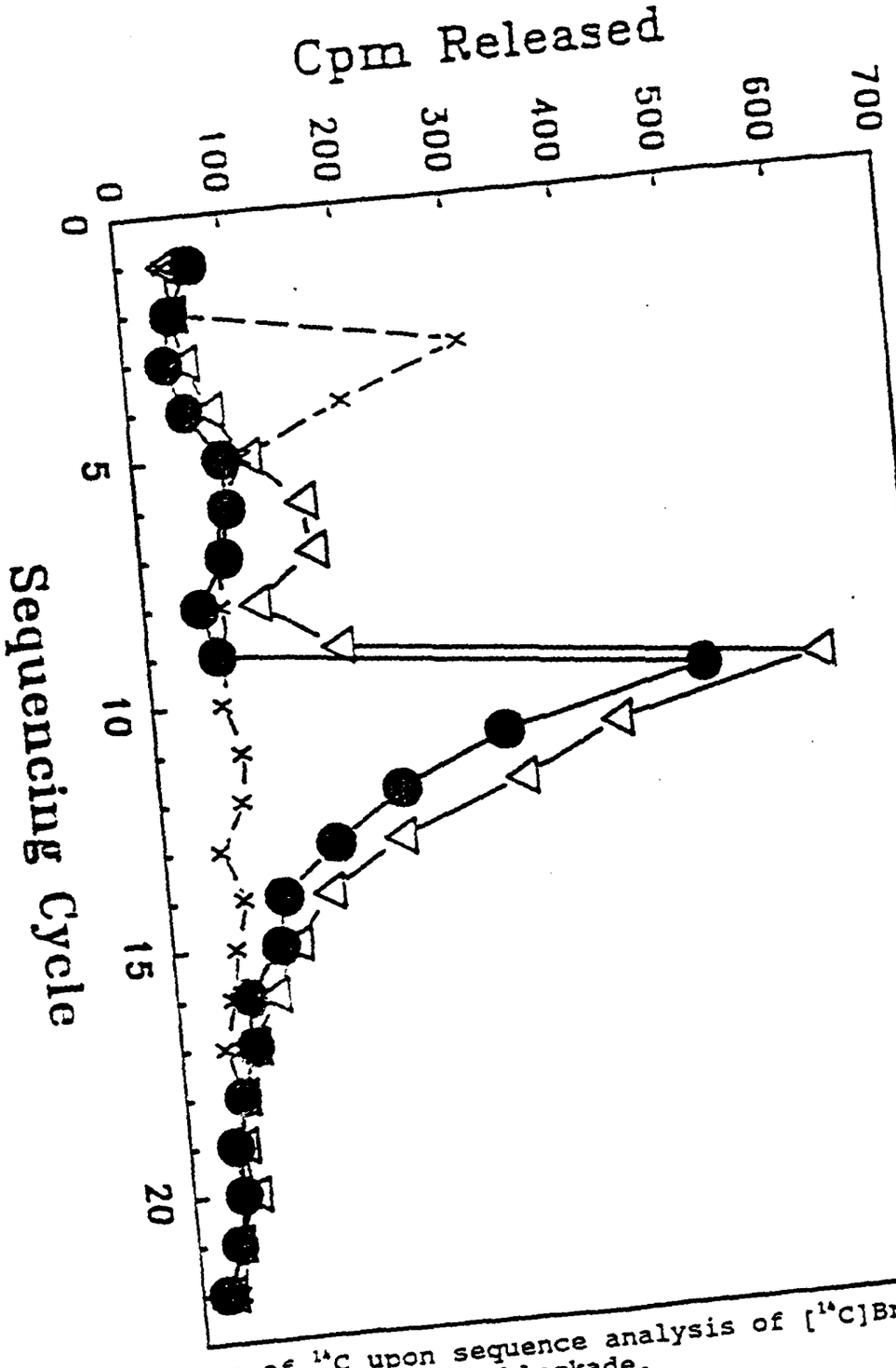


Figure 5. Release of ^{14}C upon sequence analysis of [^{14}C]BrPin-labeled tryptic peptide: OPA blockade.

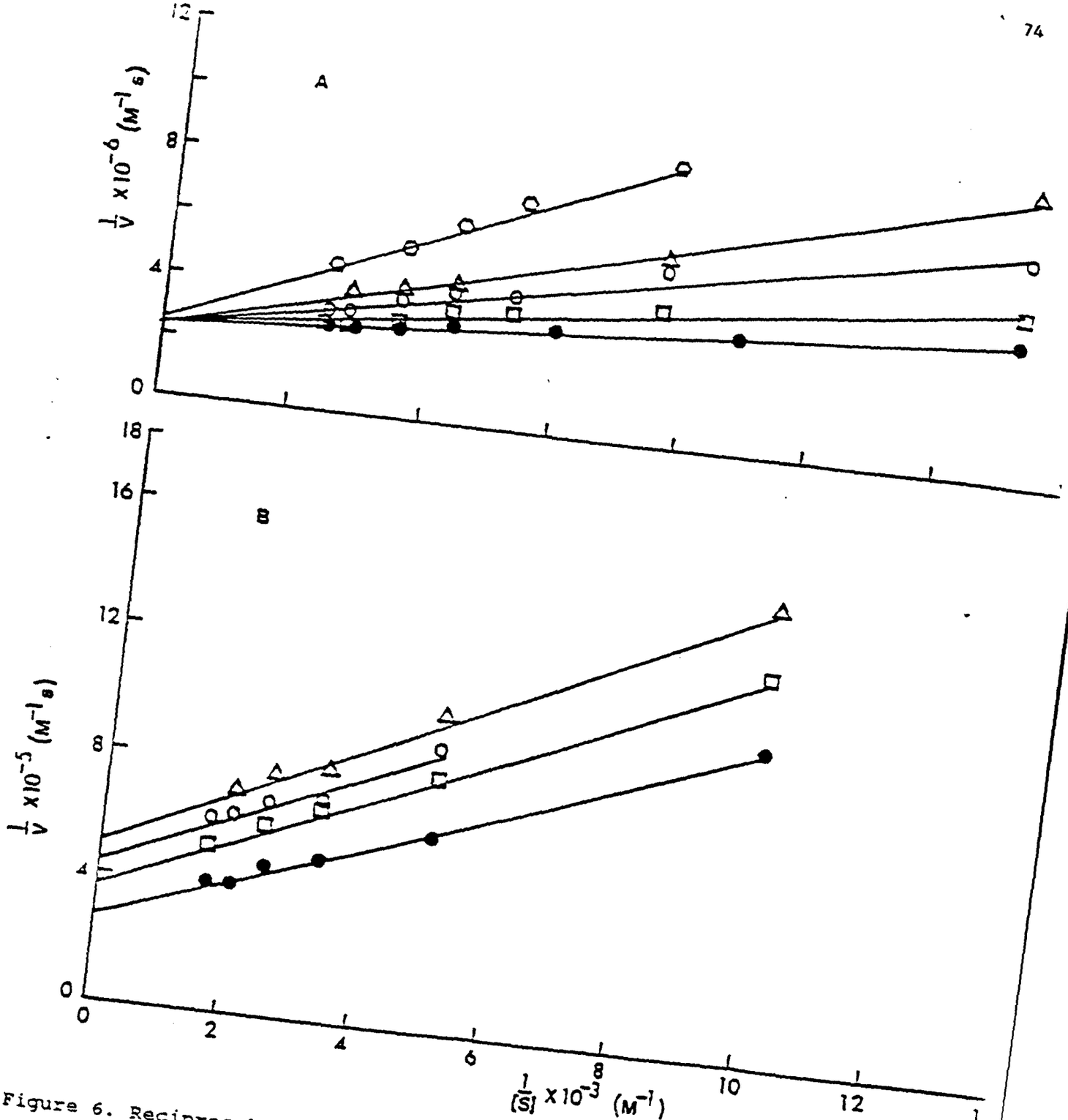


Figure 6. Reciprocal plots for reversible inhibition by 2-PDS of hydrolysis of AcCh by AcChE.

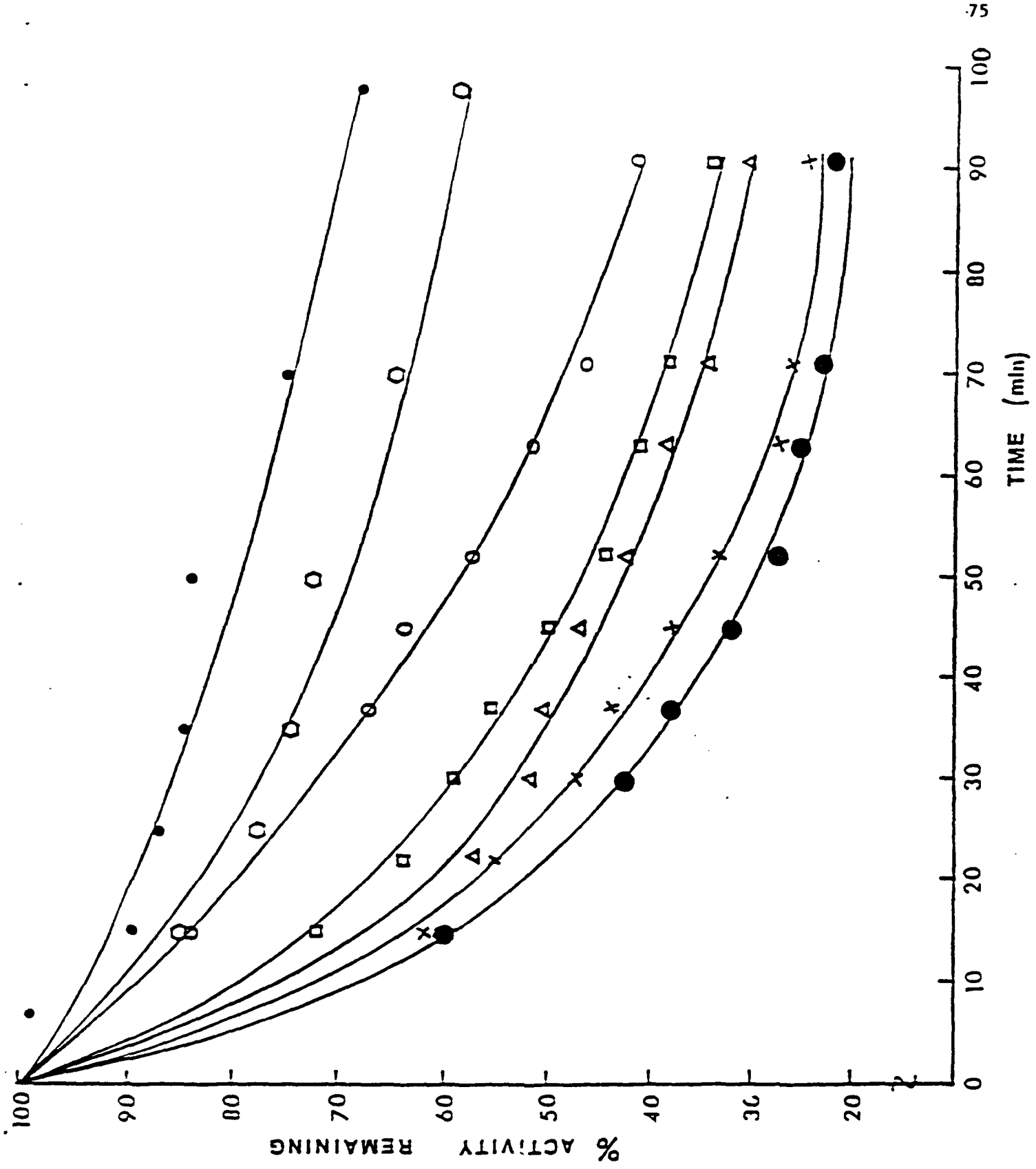


Figure 7 Irreversible inactivation of AcChE from T. nobiliana by 2-PDS.

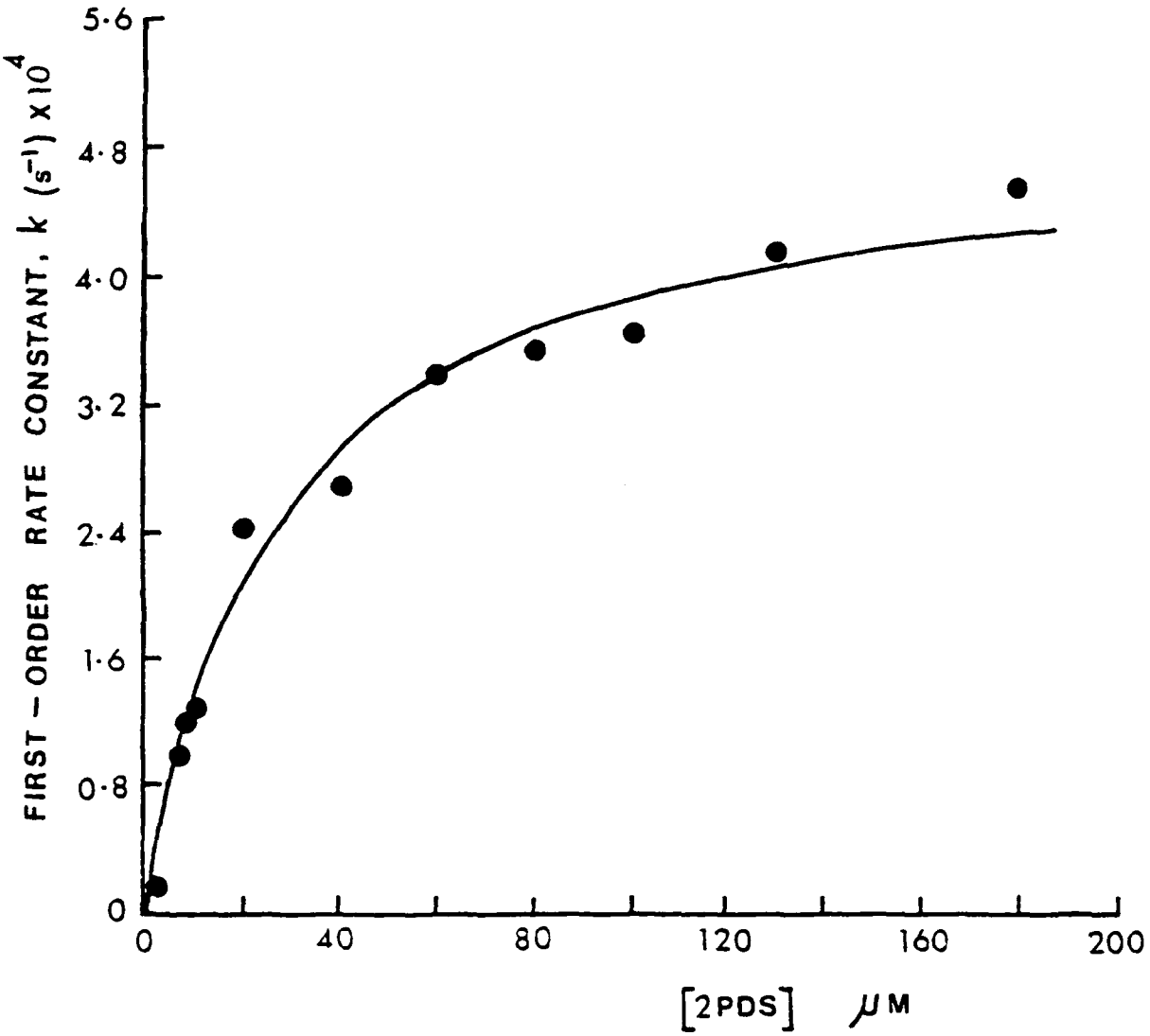


Figure 8. First-order rate constants, k, eq. 5, for inactivation by 2-PDS of AcChE from T. nobiliana.

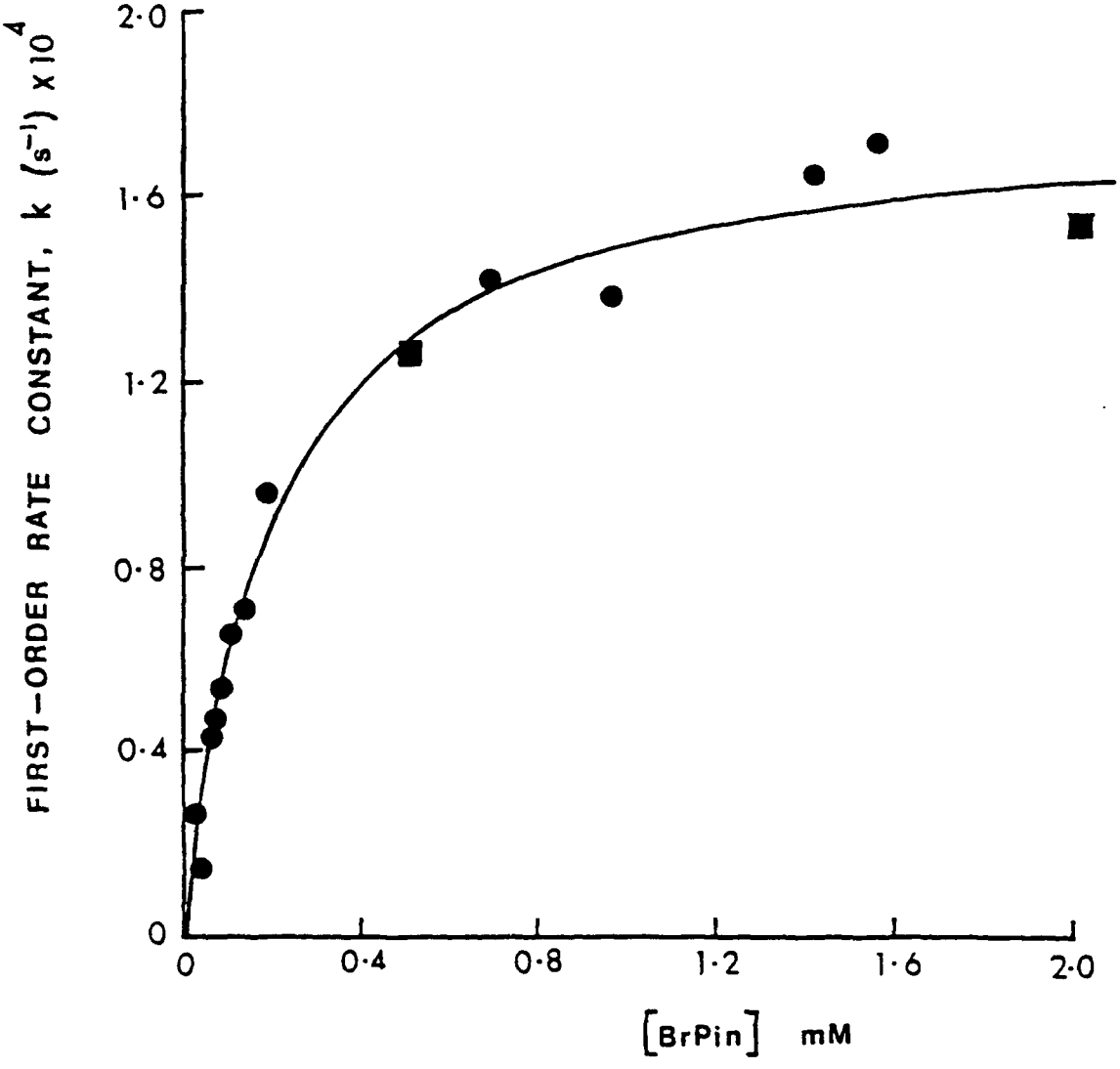


Figure 9. First-order rate constants, k, eq. 5, for inactivation by BrPin of AcChE from T. nobiliana.

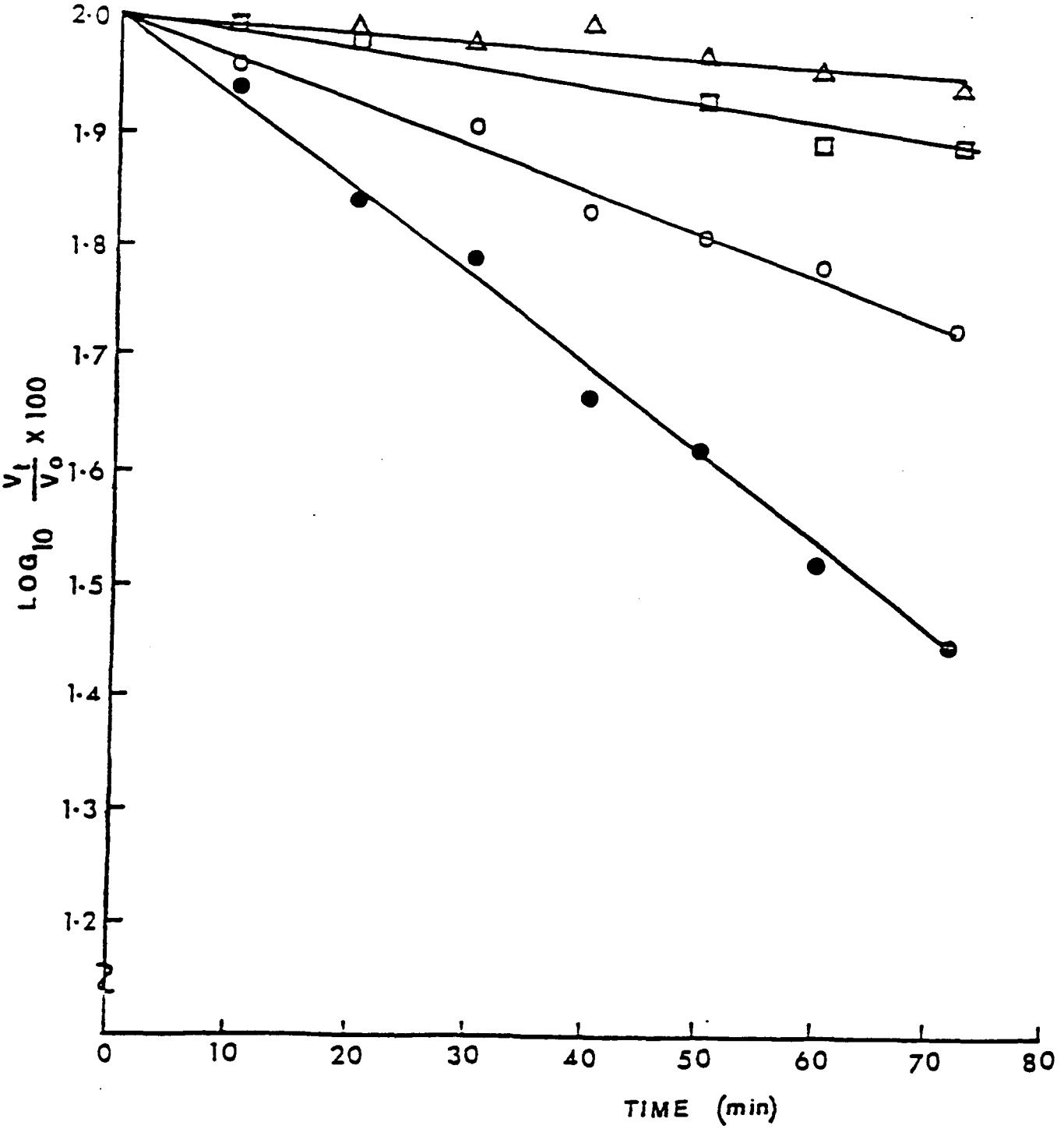


Figure 10. Retardation by TAP of inactivation of 0.10 μM AcChE from T. nobiliana by 56 μM 2-PDS, pH 7.8, phosphate buffer, 22°C.

Figure 11. Reciprocal plot for reversible inhibition by phenacyl bromide (PhABr) of hydrolysis of AcCh by AcChE.

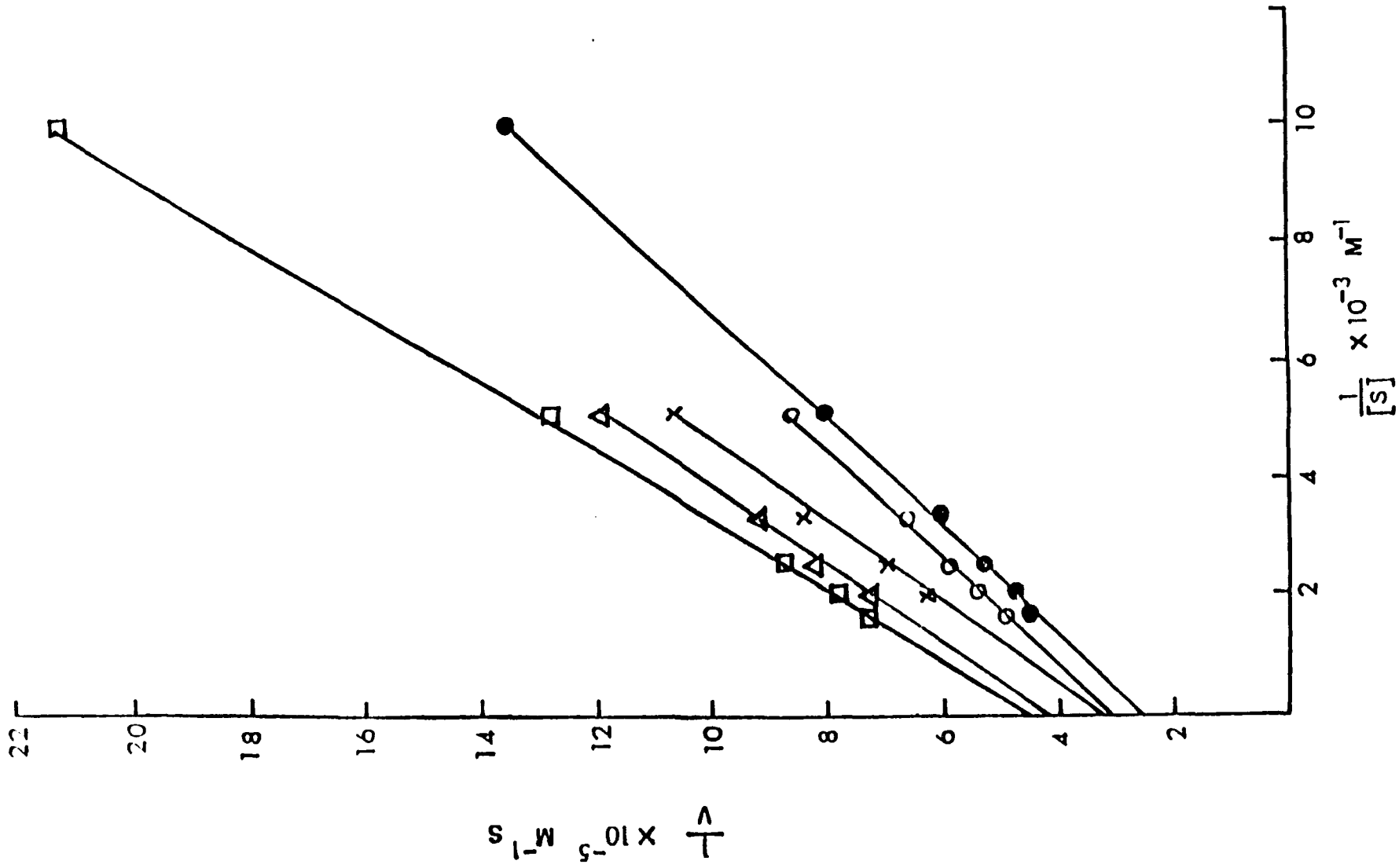
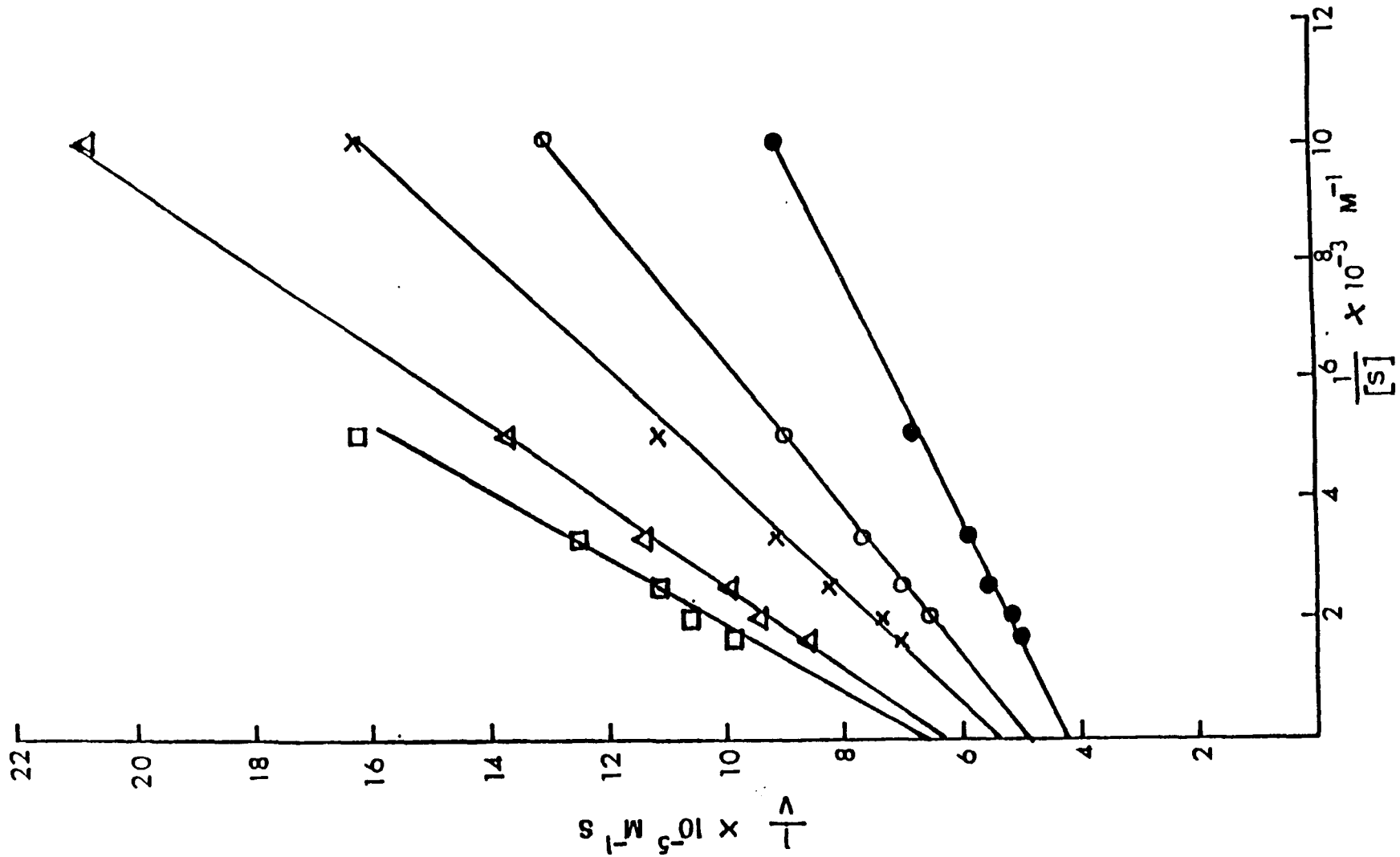


Figure 12 Reciprocal plots for reversible inhibition by methyl benzenesulfonate (MBS) of hydrolysis of AcCh by AcChE.



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1.	Dr. Saul G. Cohen,	PI
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3.	Dr. Sachiko Howard	July 1, 1987 - August 31, 1990
4.	Dr. Erdjan Salih	July 1, 1987 - December 31, 1991

GLOSSARY OF ABBREVIATIONS

AcCh	Acetylcholine
AcChE	Acetylcholinesterase
AcPy	Acetylpyridine
AcSCh	Acetylthiocholine
n-BAc	n-Butyl acetate
BCA	Bicinchoninic acid
[^{14}C]BrPin	1-Bromo-2-[^{14}C]-pinacolone
[^{14}C]Br_2Pin	,-Dibromo-2-[^{14}C]-pinacolone
BAPy	Bromoacetylpyridine
BSCl	Benzenesulfonyl chloride
BuChE	Butyrylcholinesterase
BuPy	Tertiary butyl pyridine
DEBAAc	2-N,N-Diethyl-N-butylammonioethyl acetate
DEBAP	5-N,N-Diethyl-N-n-butylammonio-2-pentanone
DeMe	Decamethonium
[^{3}H]DFP	1,3-[^{3}H]Diisopropyl fluorophosphate
DMAPy	Dimethylaminopyridine
DMBAc	3,3-Dimethylbutyl acetate
DMMPy	3-Dimethylamino-N-methylpyridinium ion
DTNB	5,5′-dithiobis(2-nitrobenzoic)acid
DTT	Dithiothreitol
EA	Acetylenzyme
EA·I	Acetylenzyme, inhibitor bound in active site
EA·S	Acetylenzyme, substrate bound in active site as inhibitor
E·S	Enzyme-substrate complex
E·S·I′	Enzyme-substrate complex, inhibitor bound in peripheral site
E·S·S′	Enzyme-substrate complex, second substrate bound as an inhibitor in peripheral site
GLC	Gas-liquid chromatography
HPLC	High performance liquid chromatography

GLOSSARY OF ABBREVIATIONS CONTINUED

IAc	Iodoacetic acid
IAm	Iodoacetamide
M7CQ	N-Methyl-(7-dimethylcarbamoxy)quinolinium ion
M7HQ	N-Methyl-7-hydroxyquinolinium ion
MAAc	2-(Methylammonio)ethyl acetate
MAcr	N-Methylacridinium
MBS	Methyl benzenesulfonate
MR	Molar refraction
MSAc	Methylsulfonylethyl acetate
NMPy	N-Methylpyridinium iodide
OPA	Ortho-phthalaldehyde
2-PDS	2,2'-Dipyridyldisulfide
PhABr	Phenacyl bromide
PMc	Phenylmercuric chloride
PTA	Phenyltrimethylammonium ion
PTH	Phenylthiohydantoin
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
TAP	5-Trimethylammonio-2-pentanone
TeMA	Tetramethylammonium ion
TFA	Trifluoroacetic acid
TMAAPh	3-Trimethylammonioacetophenone
TMAP	3-Trimethylammoniophenol
TMB-4	(1-1-Trimethylene-bis-(4-hydroxyiminomethyl pyridinium) bromide
TrMA	Trimethylammonium ion